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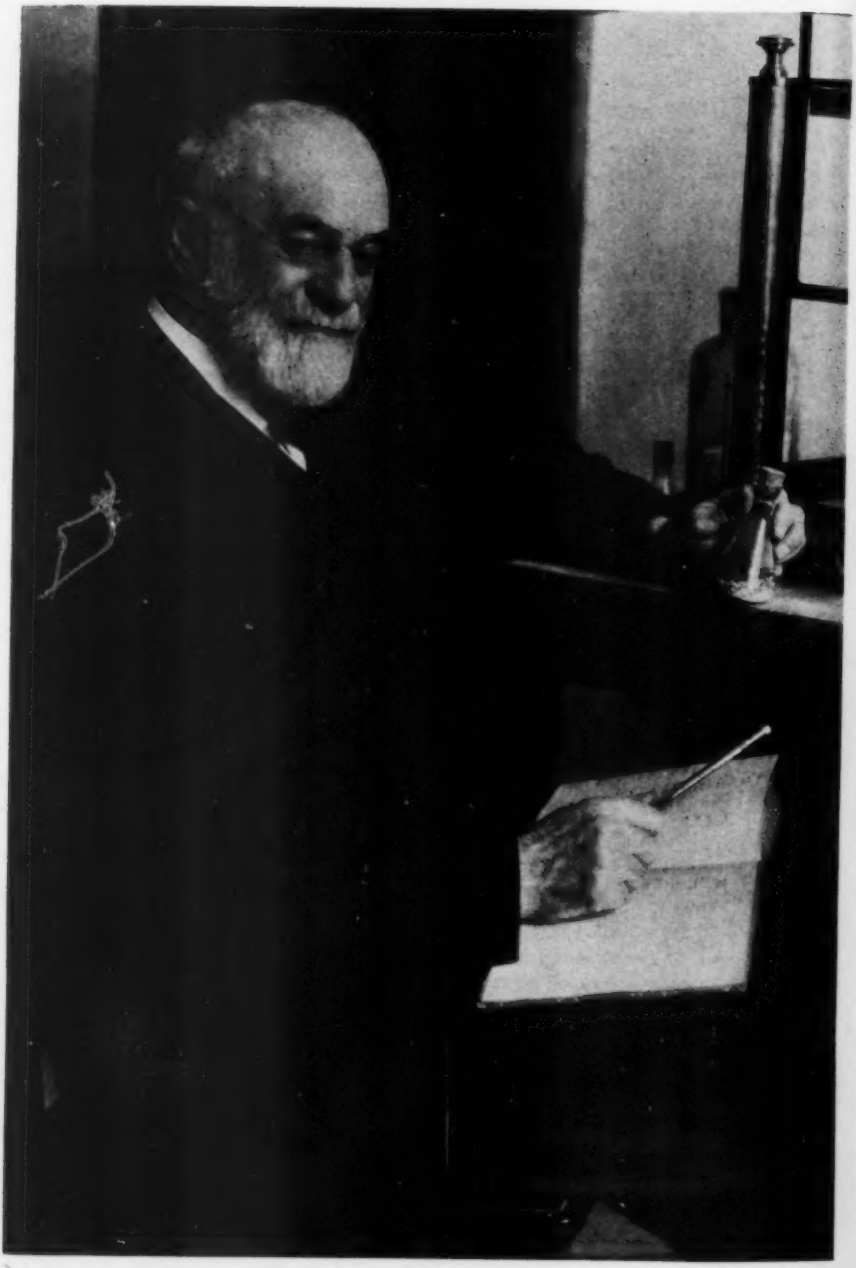
*STEPHEN MOULTON BABCOCK*

*1843–1931*



## STEPHEN MOULTON BABCOCK

(October 22, 1843 – July 2, 1931)

With this first number of volume thirty-seven a new picture of a distinguished scientist graces the front cover of the Journal, that of Stephen Moulton Babcock. He was one of our American pioneers in agricultural and nutritional biochemistry. The photograph from which the picture on the cover was taken serves as a frontispiece for this volume.

Dr. S. M. Babcock was born at Bridgewater, New York, October 22, 1843, and died in Madison, Wisconsin, July 2, 1931. On October 27, 1896, he was married to May Crandall, but no children were born of this union. His father was Pelig Babcock and his mother Cornelia Scott.

He took his A.B. degree at Tufts College in 1866 and in 1901 received an honorary LL.D. from the same institution. In 1879 he received his Doctor's degree at the University of Göttingen, Germany. From 1875 to 1877 and from 1881 to 1882 he was instructor in chemistry at Cornell University and from 1882 to 1888 chemist at the Agricultural Experiment Station in Geneva, New York. In 1888 he became Professor of Agricultural Chemistry at the University of Wisconsin and Chief Chemist of the Wisconsin Agricultural Experiment Station. He became Assistant Director of that institution in 1889.

Many honors came to Dr. Babcock. He received a bronze medal from the Wisconsin Legislature in 1899; Grand Prize of the Paris Exposition in 1900; Grand Prize from the St. Louis Exposition in 1904, a silver medal from Stockholm in 1908; and the Capper Award for distinguished service to American agriculture in 1930.



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Dr. Babcock's grasp of science was thorough, for he brought to his problems a deep understanding of mathematics, physics, and chemistry.

In the popular mind Dr. Babcock will be remembered longest for his invention of the milk fat test which bears his name. This was done in 1890 when he was 47 years of age. He has told me many times that his real contribution to the development of the milk fat test lay in his introducing the centrifuge as a part of the test, thereby shortening the time of operation. Here again his fundamental knowledge of physics stood him in good stead. Probably no contribution to agriculture by a scientist has helped more to gain the respect and confidence of farmers for agricultural experiment stations than the Babcock test. It made possible payment for milk on an equitable and impartial basis, and removed in a single stroke the obstacle that had so long stood in the way of utilizing factory methods in the manufacture of butter and cheese. The Babcock test permitted the accurate and easy determination of the fat production of individual cows, which made possible advanced registry, testing, and dairy herd improvement associations.

This inventive genius also developed a viscosimeter, the principle of which is the basis of the modern viscosimeter. Another important piece of work contributed by Dr. Babcock was his solution of the problem of metabolic water, a fundamental question in plant and animal physiology. This research is now considered a classic by plant physiologists and to animal physiologists it explains how a clothes moth can live on dry clothes and produce larva containing 75% water. It was published in Research Bulletin No. 22 of the Wisconsin Agricultural Experiment Station, March, 1912.

The present century has seen a great development in our knowledge of animal nutrition. Dr. Babcock's contribution to that development is not generally known. He lighted the torch for others to carry on. Dr. Paul de Kruif, author of Microbe Hunters, tells that story in his book Hunger Fighters. When

chemist at the Geneva Agricultural Experiment Station, 1882–1888, Dr. Sturtevant, the Director of the Station, wanted him to make some of the conventional analyses then, and still, in vogue for studies on foodstuffs. The work involved not only the analysis of the food but also the analysis of the solid excrement of a cow in a metabolism experiment. After he made the analyses and reduced the results to an ash free basis, the composition of the food was much like that of the solid excrement. From that time on he lost faith in the ability of the prevailing methods of food analysis to give valuable information about the nutritive value of a foodstuff. He also had little faith in the then current notion that the energy of a foodstuff would measure its nutritive value. He delighted in telling Atwater, Armsby or Jordan — champions of the idea that the energy of a food measured its nutritive value — that if energy was the measure, then hot water or coal should be the most excellent of foods. When he came to Wisconsin he put his ideas to work in testing with cows rations which were alike according to conventional methods of food analysis and energy content but selected from different sources, with marked differences in the resultant milk production and behavior of the animals. His notes were incomplete, through no fault of his own, and so he never published the data. That work was really the forerunner of the development at Wisconsin of the newer approach to nutrition and the first experiment, so as far as I know, using the biological method for testing the nutritive value of foodstuffs. It was a new idea and Dr. Babcock was father of it. Others have carried it on.

More than 20 years of Dr. Babcock's later scientific career were spent in fundamental work in the field of physics, especially on the constitution of matter. Even in his earlier years he had given much thought to this subject and had formulated in his own mind an hypothesis that departed radically from the commonly accepted ideas concerning the relation of matter and energy. About 1896 he began a series

of experiments in which he weighed a large mass of water on a very delicately constructed balance to study the variations in the weight of the water in solid and liquid states. As was his custom, he constructed the entire apparatus with his own hands. The persistence with which he carried on these studies, even at the advanced age of 87, inspired the admiration of all his colleagues.

The later years of his life were spent in reducing his observations on matter and energy to written form, but he would not publish on the subject during his lifetime. After his death the manuscript which he left was critically examined by a number of prominent physicists and mathematicians, not only at Wisconsin but elsewhere. It was the general consensus that the work was more qualitative than quantitative and that the conclusions drawn were not sufficiently definite to warrant publication. Some expressed the opinion that many of the basic principles now recognized in the new science of physics were foreshadowed in the bold postulates that Babcock set forth in these early studies.

Dr. Babcock left no long list of contributions to scientific literature, for he was as sparing a writer as a speaker. For the most part he played a lone hand. The freshness of his ideas came from long brooding over a subject. His inquisitive mind was always subjecting the observed fact to more crucial experimentation.

Modesty and simplicity in practically everything were characteristic of Dr. Babcock. He was a joyous comrade, a friend beloved beyond measure. He pursued the most painstaking research as if he were playing a game. He brought laughter into the laboratory for there was about him that deceptively careless air which creative spirits have as they go about their business. He brought to his tasks that gaiety of spirit which authentic greatness can afford.

Wisconsin's President Frank said of him:

"This grand old doctor of science was himself greater than anything he did, and thus he gives to us, the legatees

of his spirit, a goal toward which to point the education and the science of our time.

"This merry man of many years was made of the stuff that gives mankind its saints and martyrs. But he was a saint without seriousness and he could have gone to martyrdom without a murmur of self pity, as part of the day's work. For his was a casual greatness."

E. B. HART

# CORNEAL CHANGES IN THE RAT WITH DEFICIENCIES OF PANTOTHENIC ACID AND OF PYRIDOXINE[1]

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NINE FIGURES

(Received for publication August 23, 1948)

With deficiencies of any of a number of essential nutrients which are important constituents of the corneal tissue, vascularization and other tissue changes may occur. Changes of this type have been observed in deficiencies of protein or of any of the indispensable amino acids (Sydenstricker et al., '47). Berg and coworkers ('47) suggested that with methionine deficiency the nourishment of the avascular corneal tissue becomes inadequate and an attempt is made by the tissue to compensate for the inadequacy by the development of capillaries to bring additional nourishment. This explanation might well apply to all nutritional deficiencies where such changes appear.

Wolbach and Howe ('25) described vascularization of the cornea in rats deficient in vitamin A, but regarded this as a phenomenon secondary to the hyperkeratinization of the corneal epithelium. Bessey and Wolbach ('39), on reviewing the material used in the previous study, were disposed to discard inflammation as an explanation of this vascularization. After a careful study of the corneal changes in riboflavin deficiency Bessey and Wolbach concluded that "vasculariza-

[1] A preliminary report of this work has been published (Bowles, '47).

tion of the cornea of the rat in the absence of antecedent pathology is probably a specific and most reliable criterion of riboflavin deficiency." The vascularization was accompanied in the advanced stages by leucocytosis and marked changes of the corneal epithelium. Their "control rats subjected to the following conditions did not develop corneal vascularization: $B_1$ deficiency, $B_6$ deficiency, fasting, old age, maintenance on the experimental diet plus riboflavin." György ('42) reports seeing no corneal vascularization in rats fed a diet lacking $B_6$. Singal, Sydenstricker and Littlejohn ('47) observed no corneal abnormalities in rats deficient in nicotinic acid.

It appears probable that there are unknown factors concerned in the prevention of corneal vascularization. György ('42) states that "keratitis and vascularization of the cornea were frequently encountered in rats that received vitamin $B_1$, riboflavin, vitamin $B_6$ and pantothenic acid as supplements to their vitamin-B-free diet." There is evidence that unknown factors are also involved in preventing corneal vascularization in man. Machella and McDonald ('43) studied a series of 20 cases where the patients appeared to have the symptoms of ariboflavinosis including, in some instances, corneal vascularization. However, the patients did not improve on administration of riboflavin. In some of 4,000 R.A.F. personnel studied by Lyle, McRae and Gardiner ('44) the corneal vascularization observed seemed to be influenced more by other factors present in fruits or vegetables than by riboflavin. These data led to a consideration of the possibility that corneal vascularization might result from deficiencies of B vitamins other than riboflavin.

## EXPERIMENTAL

### *Pantothenic acid deficiency*

In an attempt to produce corneal vascularization in pantothenic acid deficiency, 27 Wistar strain rats of various ages from 7 litters were placed on a pantothenic acid-deficient diet, as shown in table 1. The diet consisted of "vitamin-free"

TABLE 1

*Corneal vascularization and growth of pantothenic acid-deficient and of control rats*

| LITTER NO. | AGE WHEN PLACED ON DIET | RATS ON DEFICIENT DIET | RATS ON CONTROL DIET | RATS WITH CORNEAL VASCULARIZATION | TIME OF [1] APPEARANCE | AVERAGE WEIGHT CHANGE |
|---|---|---|---|---|---|---|
| | *days* | | | | *days on diet* | *gm* |
| 1 | 22 | 5 | 0 | 0 | .. | + 10 |
| 2 | 41 | 5 | 0 | 3 | 59 (43–74) | + 43 |
| 3 | 50 | 6 | 0 | 4 | 71 (59–82) | — 3 |
| 4 | 52 | 3 | | 3 | 61 (51–80) | + 32 |
| | 52 | | 3 | 0 | | + 122 |
| 5 | 61 | 3 | | 0 | .. | + 2 |
| | 61 | | 3 | 0 | .. | + 95 |
| 6 | 61 | 3 | | 1 | 41 | — 14 |
| | 61 | | 3 | 0 | .. | + 125 |
| 7 | 61 | 2 | | 2 | 102 (98–105) | — 36 |
| | 61 | | 1 | 0 | .. | + 111 |
| Summary | 22–61 | 27 | | 13 | 68 (41–82) | + 9 |
| | 52–61 | | 10 | 0 | | + 114 |

[1] The figures given are mean values and the range. Since biomicroscopic examinations were made at weekly intervals, the actual time of appearance of vascularization might be from one to 6 days earlier than these figures indicate.

casein [2] 20 gm, salt mixture [3] 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. Ten control rats from the same litters were placed on a control diet which was identical with the deficient diet except that it contained 2 mg of calcium pantothenate in each 100 gm. The care and treatment of the animals were as described in an earlier paper (Bowles et al., '46).

[2] Labco.

[3] The salt mixture was the same as that used by McKibben, Madden, Black and Elvehjem ('39).

Weekly biomicroscopic examination of the eyes of the first litter of rats which was placed on the diet when 22 days of age failed to show any significant change up to the time of death. These rats lived for 43, 48, 50, 76 and 89 days, respectively, on the deficient diet.

Thirteen of the 22 older rats on the deficient diet developed definite corneal vascularization, while 5 of the 22 died before sufficient time had elapsed for corneal vascularization to appear. The corneal capillaries which developed in rats fed the deficient diet were numerous and large with prominent anastomoses as contrasted with the finer network of vessels seen in riboflavin deficiency. The appearance of the capillaries in the cornea was preceded by a thickening of the cornea and by a diffuse or nebular opacity of slight degree. The opacity increased until at the time of death the cornea was nearly opaque and was frequently ulcerated (fig. 4). Figures 1 to 5 show varying degrees of these corneal changes. The patterns of development of the capillaries were similar to those previously described as occurring in other deficiencies (Bowles et al., '46). The corneal vascularization as seen with the biomicroscope and in the injected corneas is somewhat similar to that observed in early vitamin A deficiency (Bowles et al., '46) and in deficiencies of isoleucine or valine (Sydenstricker and others, '47).

Three of the rats with varying degrees of corneal changes were placed on the control diet and, in addition, were given 1 mg per day of calcium pantothenate. Within a few days the cornea became clear and the vessels assumed a beaded appearance and gradually became bloodless, beginning at the distal end. The time required for the vessels to become nonfunctional varied from a few days to several weeks depending on their size. Careful examination of the cornea with the biomicroscope and with the dissecting microscope then showed empty capillaries still present. In the limbic area short "stumps" filled with blood could still be seen (figs. 6 and 7).

None of the control rats showed any significant corneal changes.

In general, the rats on the deficient diet gained weight for a time and then lost weight at an accelerating rate for a period preceding death. The over-all weight changes during the course of the experiment are shown in table 1.

### *Pyridoxine deficiency*

In order to study the ocular changes in pyridoxine deficiency, rats from 8 litters were placed on a pyridoxine-deficient diet [4] or on a control diet,[5] as shown in table 2. The techniques used and the details of the care of the animals in this study were described by Bowles and coworkers ('46). Weekly biomicroscopic examinations for ocular changes were made.

Up to the time of death of the rats placed on the deficient diet when 22 days of age no ocular changes had been observed. This is in accord with the observations of Bessey and Wolbach ('39) and of György ('42). The litter of rats first fed the deficient diet when 65 days of age developed no very marked symptoms of the deficiency and no ocular changes could be seen. Eleven rats of the 23 which were placed on the deficient diet when 45–57 days of age developed definite corneal vascularization.

The corneal capillaries were of a heavy branched type and their appearance was associated with a moderate opacity of the cornea (figs. 8 and 9). The corneal changes were not as extensive as in pantothenic acid or riboflavin deficiencies and the animals usually died soon after the corneal changes appeared.

In two animals with corneal vascularization which were changed to the control diet, a regression of the vascularization resulted similar to that described above for pantothenic acid deficiency.

[4] The pyridoxine-deficient diet consisted of vitamin-free casein (Labco) 20 gm, salt mixture (as used by McKibben et al., '39) 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, calcium pantothenate 2.0 mg and sufficient sucrose to make 100 gm. The control diet contained, in addition, 0.4 mg of pyridoxine hydrochloride.

[5] See footnote 4.

One of the 20 patients with symptoms of nutritional deficiency studied by Machella and McDonald ('43) improved after administration of pyridoxine. However, these investiga-

TABLE 2

*Corneal vascularization and growth of pyridoxine-deficient and of control rats*

| LITTER NO. | AGE WHEN PLACED ON DIET | RATS ON DEFICIENT DIET | RATS ON CONTROL DIET | RATS WITH CORNEAL VASCULARIZATION | TIME [1] OF APPEARANCE | AVE. WT. CHANGE |
|---|---|---|---|---|---|---|
| | *days* | | | | *days on diet* | *gm* |
| 1 | 22 | 6 | 0 | 0 | | + 7 |
| 2 | 45 | 4 | | 1 | 88 | + 13 |
| | 45 | | 2 | 0 | | + 193 |
| 3 | 48 | 3 | | 2 | 56 (56–56) | + 15 |
| | 48 | | 2 | 0 | | + 125 |
| 4 | 53 | 3 | | 0 | | + 24 |
| | 53 | | 3 | 0 | | + 204 |
| 5 | 54 | 6 | 0 | 4 | 69 (43–78) | + 26 |
| 6 | 55 | 4 | | 2 | 87 (65–108) | + 63 |
| | 55 | | 3 | 0 | | + 73 |
| 7 | 57 | 3 | | 2 | 115 (108–122) | + 83 |
| | 57 | | 1 | 0 | | + 92 |
| 8 | 65 | 6 | 0 | 0 | | + 58 |
| Summary | 22–65 | 35 | | 11 | 80 (43–122) | + 35 |
| | 45–57 | | 11 | 0 | | + 142 |

[1] The values given are the mean values and the range. Since biomicroscopic examinations were made at weekly intervals, the actual time of appearance of vascularization might be from one to 6 days earlier than these values indicate.

TABLE 3

*Corneal changes observed in rats in various vitamin deficiencies* [1]

| DEFICIENCY | VASCULARIZATION | OPACITY | SUPERFICIAL ULCERATION |
|---|---|---|---|
| Vitamin A | ++++ | ++++ | ++++ |
| Riboflavin | +++ | ++++ | |
| Pantothenic acid | +++ | +++ | ++ |
| Pyridoxine | ++ | ++ | |

[1] The corneal changes summarized here were described by Bowles et al. ('46) and in the present paper.

tors did not report the observation of any ocular changes in this particular patient.

## DISCUSSION

Deficiencies of any of 4 vitamins may result in corneal changes in the rat. The extent of these changes as observed in this and a previous study is shown in table 3. This may be compared with a similar table in which Hall and coworkers ('48) indicate the degree of corneal and lenticular change in protein and amino acid deficiencies. Corneal changes of this type are not invariably observed in all nutritional deficiencies but presumably result from deficiencies of nutrients which are of primary importance in the structure and metabolism of the cornea.

## SUMMARY

1. Heavy vascularization, thickening and opacity of the cornea were observed in 13 of 22 rats which were placed on a pantothenate-deficient diet when from 41 to 61 days of age.

2. Similar but less marked changes were seen in 11 of 23 rats placed on a pyridoxine-deficient diet when 45 to 57 days of age.

3. The changes observed were similar to those observed in early vitamin A deficiency and in deficiencies of isoleucine and of valine.

## ACKNOWLEDGMENT

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# PLATES

## PLATE 1

### EXPLANATION OF FIGURES

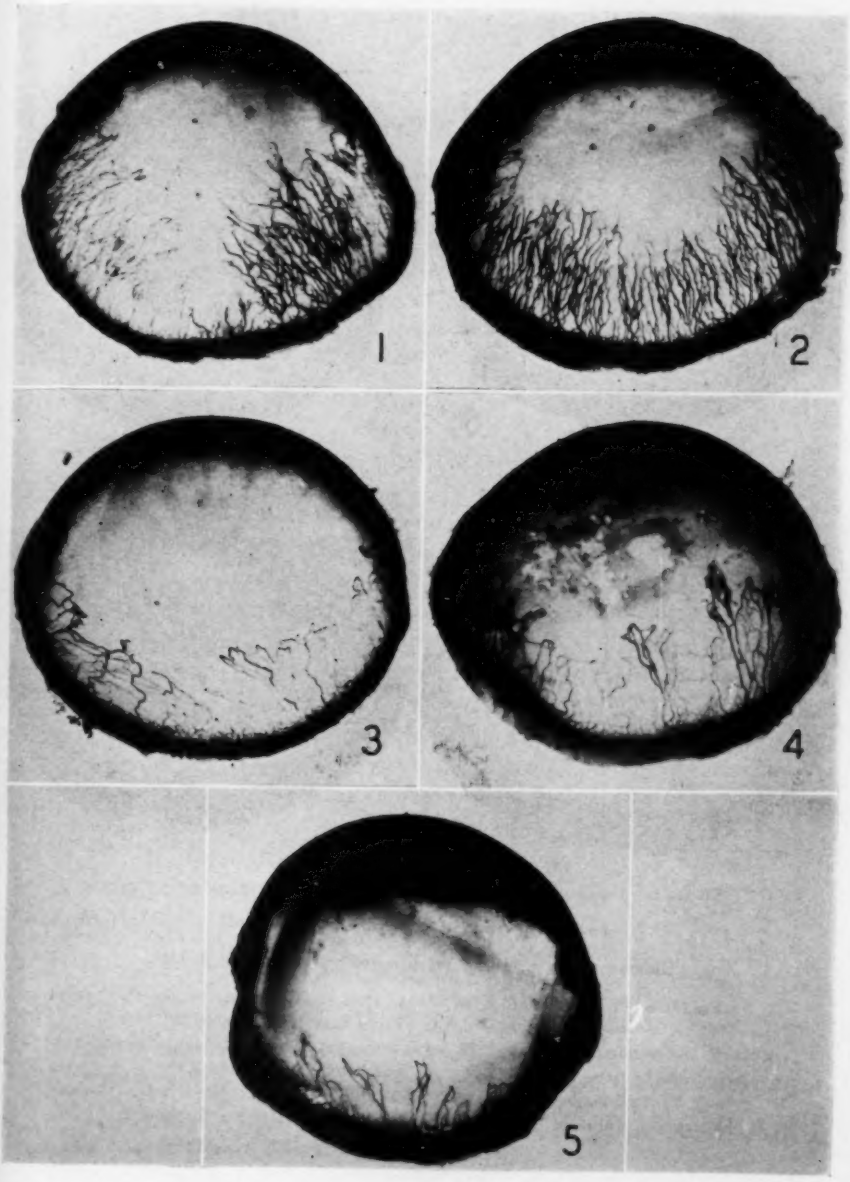
(All 18 × reduced approximately one-third)

Oblique views of India ink-injected corneas of rats fed a pantothenate-deficient diet.

1 and 2 Both corneas of rat from litter no. 4, table 1. Corneal vascularization observed after 51 days; rat killed after 76 days on the diet.

3 and 4 Both corneas of rat from litter no. 3, table 1. Corneal vascularization observed after 59 days; rat killed after 104 days on the diet.

5 Cornea of rat from litter no. 3, table 1. Corneal vascularization observed after 80 days; rat killed after 93 days on the diet.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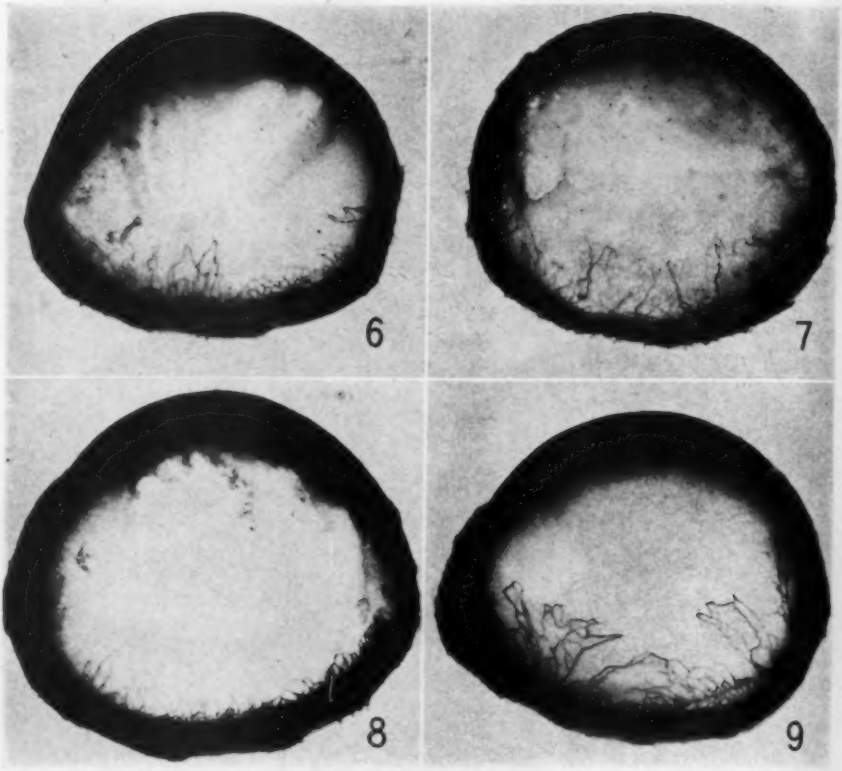


(All 18 × reduced approximately one-third)

Oblique views of India ink-injected corneas from 4 rats.

6 Pantothenate-deficient diet 87 days, then control diet 19 days. Corneal vascularization observed after 74 days. (From litter no. 2, table 1).

7 Pantothenate-deficient diet 97 days, then control diet 22 days. Corneal vascularization observed after 60 days. (From litter no. 2, table 1.)

8 and 9 Pyridoxine-deficient diet 171 and 96 days, respectively. Corneal vascularization observed after 78 days. (Both from litter no. 5, table 2.)

# MOUSE GROWTH ASSAY PROCEDURES FOR THE "ANIMAL PROTEIN FACTOR"[1]

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FIVE FIGURES

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It has been well established that there is associated with animal protein materials an unidentified factor, or factors, not present in yeast or the major seed protein concentrates that is essential for normal growth and may also be necessary for the maintenance of life. The existence of such a factor has frequently been suggested by many workers. However, differences in the composition of the basal diets and in the degree of depletion of the test animals employed by different investigators have resulted in considerable confusion, not only with regard to the number of factors involved but also with regard to the nature of the deficiency symptoms to be expected.

This investigation was designed to develop a convenient assay procedure for the "animal protein factor" and to study, in part, its role in animal nutrition.

## EXPERIMENTAL METHOD

During the war years the stock diet of the Sharp and Dohme mouse colony consisted almost entirely of a mixture of cereal grains and vegetables. Weanling mice taken from the colony

[1] A preliminary report of this study was presented before the American Chemical Society, Chicago, Illinois, April 19, 1948.

at this time showed a marked increase in growth rate when liver was added to basal diets containing either yeast or a mixture of synthetic B vitamins or both (Bosshardt et al., '45). Following the war, when meat scraps and dry skim milk were again used in the colony stock diet, this difference no longer could be obtained. The growth of the mice receiving liver was not significantly greater than that of those receiving the unsupplemented basal diet. This suggested a storage of the "animal protein factor" in the weanling mice sufficient to meet requirements for the duration of the test period.

TABLE 1

*The effect of depletion of the "animal protein factor" on reproduction in the mouse*

| LITTER NO. | NO. FEMALES | NO. YOUNG WEANED | NO. YOUNG WEANED PER LITTER |
|---|---|---|---|
| 1 | 35 | 247 | 7.1 |
| 2 | 30 | 199 | 6.8 |
| 3 | 28 | 117 | 4.2 |
| 4 | 26 | 0 | 0 |

Thirty-five pregnant female mice that were obtained from the Sharp and Dohme colony three to 5 days before parturition were placed on an animal protein-free diet. The composition of this diet was: 10% wheat germ middlings, 40% of a well heated soy flour, 18% hydrogenated cottonseed oil,[2] 3% cod liver oil, 7% dried yeast,[3] 18% glucose,[4] and 4% salt mixture (Jones and Foster, '42). This diet supported normal growth with weanling mice taken from the stock colony. The adult female mice were maintained on this diet exclusively until 4 successive litters were obtained.

Table 1 shows the number of mice weaned (16 days of age) per litter throughout the study. This number decreased with each successive litter until the 4th litter, when none was

[2] Primex.
[3] Anheuser-Busch, strain G.
[4] Cerelose.

raised to weaning age. This was not due to a decrease in the number of mice born in the successive litters but rather to an increase in *post partum* mortality, with all the deaths occurring during the first three days after parturition. It was also noted that the death of young mice involved complete litters or else all young survived. It must be concluded that if this "all or none" type of mortality was due to a deficiency of the "animal protein factor" there were pronounced differences in the reserve stores among the female mice employed. Whether or not death of the young mice can be attributed to a lactation failure is debatable. Since some of the mice died with milk in their stomachs, it would appear that the mothers were secreting milk but that the milk was deficient in some nutrient, presumably the "animal protein factor," required by the young mice. In the light of the conclusions reached by Allgeier et al. ('48) the evidence would appear conclusive that the basal diet employed in this study was inadequate for normal reproductive cycles in the mouse.

When the mice of the first three series of litters were weaned the males were placed on two test diets. The first diet contained 30% alcohol-extracted casein,[5] 25% hydrogenated cottonseed oil, 2% corn oil,[6] 20% glucose, 4% salt mixture (Jones and Foster, '42), 2% cellulose,[7] and 17% white dextrin. The following supplements were added per 100 gm of this diet: alpha-tocopherol, 4 gm; vitamin A, 900 U.S.P. units; vitamin D, 180 U.S.P. units; 2-methyl-1,4 naphthoquinone diacetate, 1 mg; choline chloride, 200 mg; thiamine hydrochloride, 0.8 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride 0.8 mg; niacin, 4.0 mg; calcium pantothenate, 4.4 mg; para-aminobenzoic acid, 4.0 mg; and inositol, 21.6 mg. The second diet was of the same composition except that 1% of a defatted, dehydrated whole liver powder[8] was added at the expense of an equivalent amount of white dextrin.

[5] G. B. I. vitamin test.

[6] Mazola.

[7] Cellu flour.

[8] VioBin.

The weight gains that were obtained during a 15-day period are shown in table 2. Whereas the growth of the mice receiving the liver was the same for each of the three litters, there was a steady decrease in the growth rates of the mice from the successive litters that were not fed liver. These results show a positive correlation between the degree of depletion of the female mice maintained on the all-vegetable diet as determined by reproduction efficiency and the growth rates of the mice from the successive litters fed a diet containing no "animal protein factor." The growth rates of the mice receiving liver show that liver contains a substance, or substances, that correct a deficiency of this factor.

TABLE 2

*Average weight gains of mice depleted of the "animal protein factor" and fed diets with and without added liver*

| LITTER NO. | AVE. WT. GAIN WITH NO LIVER | AVE. WT. GAIN WITH 1% DRY LIVER | DIFFERENCE |
|---|---|---|---|
| | *gm* | *gm* | *gm* |
| 1 | 6.05 | 10.98 | 4.93 |
| 2 | 2.20 | 10.43 | 8.23 |
| 3 | 0.90 | 10.40 | 9.50 |

One study was made to determine the amount of liver in the diet necessary for a maximum growth response. For this study male mice obtained from the first litters of depleted females were used. Two liver preparations, a defatted, dehydrated liver powder [9] and Wilson's 1:20 liver concentrate powder, were incorporated at varying levels into the basal diet previously described and fed ad libitum to groups of 8 male weanling mice. The weight gains that were obtained are shown in figure 1. The effects of the two liver preparations were found to be the same, with a maximum effect observed at levels ranging from 0.5 to 1.5% of the diet. A diminished effect was found with the defatted, dehydrated whole liver powder when the level in the diet was increased from 1.5%

[9] See footnote 8, p. 23.

to 2%. As a result of this experiment the standard positive control employed in all subsequent work has been a diet fed ad libitum and containing 1% of the defatted, dehydrated whole liver powder.

Although weanling mice obtained from depleted females would be ideal assay animals, their use was discontinued because of the difficulties involved in obtaining sufficient numbers of uniformly depleted mice at any one time.

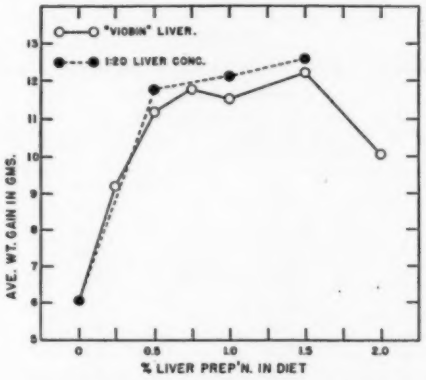


Fig. 1 The relationship between the 15-day weight gains of mice and the level of liver in the diet.

Ershoff ('47) has shown that liver contains a factor or factors distinct from all the known water soluble or fat soluble vitamins that completely counteracts the growth retardation resulting from the feeding of toxic amounts of thyroid-active materials. Preliminary experiments using an iodinated casein with thyroid activity [10] showed that similar results could be obtained with the mouse when the thyroid-active agent was incorporated into the basal diet at a level of 0.5%.

Table 3, the data of which are taken from the results of 8 successive studies, shows the 15-day growth responses of the negative and positive control groups. The mice in the negative

[10] Protamone. Cerophyl Laboratories.

control group received the "purified" basal diet previously described with the addition of 0.5% iodinated casein with thyroid activity,[11] and those in the positive control group received the same diet with the addition of 1% of a defatted, dehydrated, whole liver powder. The average increase in the growth rate attributable to the addition of liver was 62%.

It appeared that the use of the iodinated casein, or some other thyroid-active material, would make possible a relatively simple growth assay procedure. To determine whether the use

TABLE 3

*The growth responses of mice fed 0.5% iodinated casein (Protamone) with and without added liver*

| AVE. WT. GAIN WITH NO LIVER | AVE. WT. GAIN WITH 1% DRY LIVER | DIFFERENCE | INCREASE WITH 1% DRY LIVER |
|---|---|---|---|
| *gm* | *gm* | *gm* | % |
| 6.67 | 11.52 | 4.85 | 73 |
| 6.67 | 10.13 | 3.46 | 52 |
| 7.24 | 10.20 | 2.96 | 41 |
| 7.06 | 10.50 | 3.44 | 49 |
| 7.29 | 12.06 | 4.77 | 65 |
| 4.70 | 8.18 | 3.48 | 74 |
| 8.15 | 12.20 | 4.05 | 50 |
| 5.30 | 10.15 | 4.85 | 92 |
| Ave. 6.64 | 10.62 | 3.98 | 62 ± 6.0 [1] |

[1] Standard error = $\sqrt{\frac{\Sigma d^2}{N\,(N-1)}}$.

of depleted mice would be satisfactory as a test for the same activity as the use of mice fed the iodinated casein, assays using both types of assay animal were run on a series of preparations.

The samples that were used were prepared from three different sources, liver, pancreas and crude casein, and were chosen so as to give a range of potencies. The preparations were incorporated into the basal diets at identical levels and were fed to groups of 7 male weanling mice for 15 days. The depleted mice used were obtained from the second litters of

[11] See footnote 10, p. 25.

the depleted females. With each series a negative control group fed no liver and a positive control group fed 1% dehydrated, defatted liver were included. The potencies of the preparations were determined on a percentage basis, the differences in weight gains between the negative control mice and the mice receiving the preparation being compared with the differences between the negative and positive control groups. The results are shown in figure 2. A correlation coefficient of 0.89 was obtained between the two assays. Since these

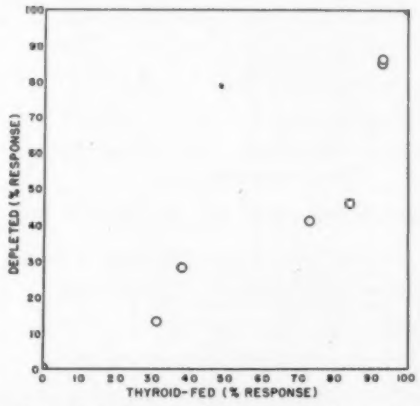


Fig. 2 A comparison of the responses of depleted mice and those fed iodinated casein (Protamone) when given "animal protein factor" preparations with varying potencies.

results suggested that the same activity was being measured by the two assay procedures, the use of mice fed iodinated casein appeared to be the better method because of its simplicity.

The growth retardation that was obtained by the addition of an iodinated casein with thyroid activity to a basal diet containing no source of the "animal protein factor" could not be obtained when a desiccated [12] or a defatted and dehydrated thyroid tissue [13] was used. The thyroid tissue may

[12] Armour and Co.

[13] See footnote 8, p. 23.

contain sufficient "animal protein factor" to counteract the growth retardation of mice attributable to thyroid activity. Another possibility is that mice and rats differ in their sus-

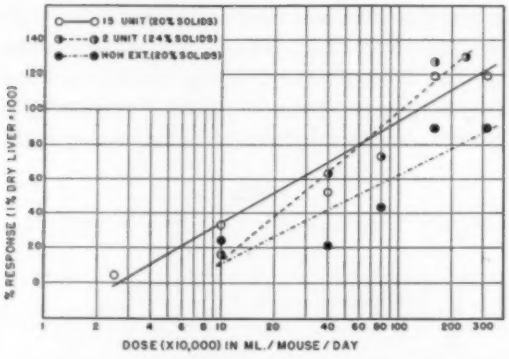


Fig. 3 The growth responses of mice fed the three liver preparations.

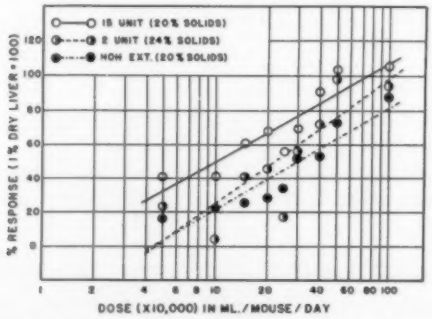


Fig. 4 The growth responses of mice given the three liver preparations by intraperitoneal injection.

ceptibility to the growth depressing effects of desiccated thyroid.

Using the mice fed iodinated casein, the relative potencies of three liver preparations were determined. These were: (1) a 15 U.S.P. unit per ml injectable liver extract; (2) a 2 U.S.P. unit per ml injectable liver extract; and (3) a water

extract of a defatted, dehydrated liver concentrated *in vacuo* to the same solid content as the two injectable extracts.

The results obtained with the oral feeding of the liver preparations for a 15-day period are shown in figure 3. The 15-unit liver extract was found to be about three times as potent as the water extract, with the two unit extract intermediate in effect.

The comparative potencies of the same three liver preparations were also determined when they were administered

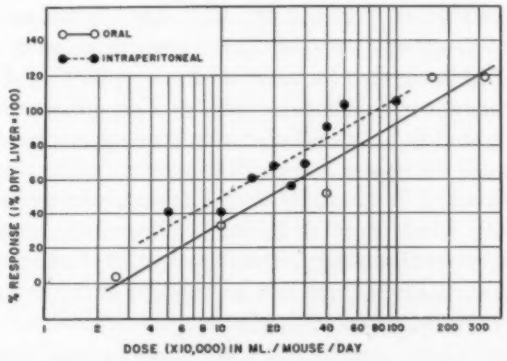


**Fig. 5 A comparison of the growth responses of mice receiving a 15-unit liver extract orally and by intraperitoneal injection.**

by intraperitoneal injection. The total dose for the 15-day period was given in two equal injections, one on the first day and the second on the 7th day of the period. The results, shown in figure 4, indicated that the same relative potencies resulted as were obtained by oral feeding. However, as is shown in figure 5, intraperitoneal administration resulted in about twice the response that was obtained when the liver extracts were given orally.

## DISCUSSION

Some early workers, employing the rat growth method for vitamin A assay, noted that the growth of rats was sub-

optimum when highly purified casein was used as the protein source. The results of Coward et al. ('29) and Guha ('31) suggested that yeast was inadequate as a source of the water soluble vitamins. An improvement in the growth rate of their rats was noted when their basal diets were supplemented with such materials as fresh milk, wheat embryo, an extract of wheat embryo, ox muscle, liver, egg, grass, lettuce, or alfalfa.

Mapson ('32, '33) carried out one of the most complete of the early studies and showed that an apparently adequate diet could be improved by the addition of liver. His studies demonstrated quite conclusively that certain animal protein sources, particularly liver, contained a water soluble substance not present in yeast that had a stimulating effect on the growth and lactation of rats. His failure to find an effect when wheat germ was added to his basal diet would suggest either that his factor, named "physin," was distinct from the factor proposed by Coward et al. ('29) or that the latter workers were dealing with a multiple deficiency. The data presented in the present report lend support to the first of these hypotheses, since unextracted wheat germ was used as a component of the animal protein-free depletion diet.

Troescher-Elam and Evans ('41) have shown that supplementation of a basal diet containing pure B vitamins and cod liver oil with liver or an extract of liver will improve the growth of male mice. Increasing the levels of casein, minerals, or B vitamins produced no effect. McIntire et al. ('43) reported that liver contains a substance necessary for the optimum growth of rats receiving the pure B vitamins. This finding was tentatively interpreted by Zucker and Zucker ('44) as indicating that the factor was associated with high quality proteins as an essential amino acid or as an impurity, since they could find no effect when the casein level in their basal diet was increased to 24% ($N \times 6.25$).

White and Sayers ('42) observed a more rapid growth rate and a higher food intake with rats fed a diet containing a pancreas derivative as the protein source than when a similar diet containing casein was fed. Their basal diet contained yeast

as the source of vitamin B. It was shown, however, by Bosshardt et al. ('45) that this increased growth rate could be attributed to a water soluble factor present in the pancreas preparation. Similar results were obtained when an extract of liver was added to the basal diet containing casein as the protein source. Bosshardt et al. further showed that the effects of liver could be enhanced by increasing the level of protein in the diet.

Hartman, Dryden and Cary ('41), Hartman and Cary ('42) and Cary and Hartman ('47) have suggested that a factor essential for the optimum growth of rats is present in liver, beef muscle, milk products, and certain grasses. Their factor, nutrient X, is not present in yeast or whole wheat, nor is it identical with any of the known vitamins. Nutrient X is presumed to be relatively heat stable.

In a study of the effects of different grains on the growth of chicks fed a soybean meal diet Whitson et al. ('45) found that wheat was superior in this respect to corn or barley. The addition of 3% sardine meal or 8% dried cow manure to the corn or barley diets appeared to supply these rations with the factor or factors responsible for the superiority of wheat over corn. These results would confirm those of Coward et al. ('29) to the effect that wheat contains an unidentified growth factor. Recently Novak and Hauge ('48) have obtained evidence that distillers' dried solubles contains a heat stable factor essential for rat growth. Prior to the work of Whitson et al., Hammond ('42, '44) showed that cow manure contains a growth promoting factor for chicks. This was confirmed by Whitson et al. ('45) and by Rubin and Bird ('46). The latter workers also demonstrated an equivalent growth promoting effect produced in chicks with Wilson's liver fraction L, which would suggest that the factor or factors in cow manure and liver are identical.

Jaffe ('46) and Jaffe and Elvehjem ('47) have shown the presence in liver of a heat labile factor which enhances the growth of rats. They could not produce any similar effect with a butanol soluble fraction of liver, which would appear to

differentiate this factor from that studied by Bosshardt et al. ('46). Jaffe and Elvehjem ('47) and Robblee et al. ('48) also have shown that fish press water (fish solubles) contains a growth factor for rats similar in many properties to the heat labile factor of Jaffe.

The results of Ershoff ('47) and Ershoff and McWilliams ('48) showed that the feeding of liver would completely counteract the retardation of growth in rats fed toxic amounts of thyroid and ovarian development in immature rats fed massive doses of alpha-estradiol. Wheat germ was without effect, whereas a slight effect was noted with yeast. Whole liver and yeast were both effective in prolonging the survival of the rats fed the thyroid-containing diets.

In a recent study Zucker and Zucker ('48) have described a factor, zoopherin, that is present in liver, crude casein, fish solubles, and cow manure and that is essential for the growth of rats fed yeast-containing plant rations. These authors suggested that zoopherin may be identical with the nutrient X of Hartman, Dryden and Cary and Rubin and Bird's cow manure factor.

The lack of complete agreement among the workers in the field of unidentified nutrients would suggest that more than one factor is involved. Other variables contributing to the confusion may be differences in the basal diets employed and differences in the degrees of depletion of the test animals used.

The evidence appears to indicate that liver contains a multiplicity of unidentified growth factors. At least one of these factors is present in wheat and another, or the same factor, is present in yeast. Liver may contain at least two factors not present in yeast, one of which is heat stable and the other heat labile. This, however, needs to be investigated further since preliminary studies in this laboratory (unpublished) indicate that the stability to heat may be dependent upon the state of purification of the materials tested.

Vitamin $B_{12}$ has recently been isolated in pure form by Rickes et al. ('48) and Smith ('48). This material has been

shown by Ott et al. ('48) to possess growth-promoting properties for chicks similar to those shown by preparations of liver and fish solubles, suggesting the possibility that vitamin $B_{12}$ may be identical with the "animal protein factor." Further work with vitamin $B_{12}$ should help to clarify the present complications with regard to unidentified growth factors.

## SUMMARY

Two mouse growth methods have been developed for the assay of the "animal protein factor." One method involves the use of growing mice born of mothers that were maintained on a diet free of this factor. The second method is based on the ability of the "animal protein factor" to counteract the growth retardation of mice that are fed thyroid-active materials.

The results of these studies showed that: (1) Liver contains a factor or factors essential for growth not present in yeast or wheat germ, nor identical with any known vitamin factor; (2) this factor counteracts the growth retardation of mice that are fed thyroid-active materials; (3) the "animal protein factor" may be transmitted from the mother to the young during gestation or lactation or both and may be stored by the animal for a considerable period of time; (4) a severe lack of the "animal protein factor" in the maternal diet and tissue stores results in a pronounced mortality of young mice one to three days *post partum*.

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# INFLUENCE OF AGE AND OF VITAMIN A INTAKE ON THE STORAGE OF VITAMIN A IN THE LIVER OF THE RAT [1]

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Those who have had extensive experience in assaying for vitamin A by the biological method have frequently observed wide variations in the time required to deplete young rats of their body store of this vitamin. This difference in the time required for depletion usually varies with young rats from different colonies, from different genetic strains, and from the same breeding stock but subsisting on different rations. However, variations in the rate of depletion of young rats from the same breeding colony and fed the same rations are also observed. Such variations sometimes appear to be seasonal and are frequently attributed to variations in the vitamin A or provitamin A content of the breeding colony diet.

There are, however, variations in the time required to deplete young rats of their body reserves of vitamin A which cannot logically be attributed directly to genetic or dietary differences. These variations are usually associated with differences in the size and in the age of the test animals when placed on the vitamin A deficient diet. That such variations have been recognized in the past is attested by the fact that the U. S. Pharmacopoeia ('47) has prescribed definite age

[1] Authorized for publication on July 31, 1948, as Paper No. 1457 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

and weight limitations for the young rats used in official assays. While the probable effects of variations in age, in body weight, and in previous vitamin A intake on the rate of vitamin A depletion have been recognized, the extent to which these variables determine the time required to deplete the test animals has not been adequately studied. The present report relates to the effects of age and of previous vitamin A intake on the amount of this vitamin stored in the body of the rat.

Since ample evidence is at hand to show that the liver is the principle vitamin A storage tissue of the rat (Osborne and Mendel, '18; Sherman and Boynton, '25; Moore, '31; Baumann et al., '34; McCoord and Luce-Clausen, '34; and others), the vitamin A content of this organ has been considered a reliable index of the vitamin A reserve of this animal.

## EXPERIMENTAL

The first phase of the investigation involved normal rats of various ages selected from our breeding colony. These rats were from breeding stock which had been reared and maintained through many generations on a complex breeding ration of our own formulation. Rats of both sexes, ranging in age from one to 749 days, were used. While Lemley et al. ('47) have shown that storage of vitamin A in the liver of the rat is not affected by sex differences, a special effort was made to determine the vitamin A content of livers from female rats which had previously weaned litters and which represented a wide range of reproductive ages. It was believed that these data would give some indication as to whether the progeny would readily respond to vitamin A depletion.

All animals except the unweaned young were weighed and sacrificed by decapitation 24 hours after their last feeding period. The abdominal cavity was opened at once and the entire liver removed, pressed between two sheets of heavy filter paper to remove blood and adhering body fluid, weighed and transferred immediately to an Erlenmeyer flask containing an appropriate volume of freshly prepared alcoholic

potassium hydroxide solution (10%), and then macerated by means of a blunt-end glass rod. The flask was attached to a reflux condenser and the contents digested on a hot water bath until the animal tissue had completely disintegrated. The digest was cooled, transferred to a separatory funnel, 100 ml of distilled water added, and the mixture extracted three times with 50 ml portions of peroxide-free diethyl ether. Previous experience had indicated that three extractions were sufficient to remove all of the vitamin A. The combined ether extract was washed free of alkali with distilled water, dried over anhydrous sodium sulfate, concentrated to an oily consistency through vacuum distillation, and the residue taken up in purified chloroform. The vitamin A content of the chloroform extract was determined by means of the Carr-Price procedure ('26) as modified by Dann and Evelyn ('38), using an Evelyn photoelectric colorimeter equipped with a no. 620 filter to measure the intensity of the color. At first the colorimeter was standardized with U.S.P. Reference Cod Liver Oil no. 3, but in the latter phases of the study the instrument was restandardized with the new U.S.P. Vitamin A Reference Standard (crystalline vitamin A acetate in cottonseed oil). All vitamin A values reported are calculated on the basis of the Vitamin A Acetate Standard. Individual determinations were run on all liver tissues except those from the one-day-old rats, in which cases the livers were pooled before weighing and saponification and the total vitamin A content of the unsaponifiable extract was determined. In all instances the vitamin A values reported are the average for all animals constituting the respective experimental groups.

A second phase of the investigation consisted of determining the relative amounts of vitamin A stored in the livers of rats as the result of feeding increasing daily doses of a natural ester concentrate of vitamin A (882,400 U.S.P. units per gram) under conditions comparable to those observed in the official U.S.P. biological assay. In this phase, a total of 27 experimental groups were used. The amount of vitamin A fed daily varied from 0.73 to 75,520 U.S.P. units. All of the

test animals were from our own breeding colony, produced under the usual breeding conditions and weaned at ages ranging from 21 to 24 days. All animals were caged in individual all-metal units during both the depletion and feeding periods. They were fed liberal quantities of a vitamin A deficient diet consisting of 18% purified casein, 4% salt mixture, 8% irradiated brewers' yeast, 5% hydrogenated vegetable oil, and 65% dextrinized corn starch. When the animals had become depleted of their body stores of vitamin A, they were assigned to experimental groups just as if they were to be used in an official vitamin A assay. Each rat was then fed daily a definite amount of the vitamin A concentrate after the concentrate had been properly diluted with refined cottonseed oil, so that the daily allotment of oily supplement was 0.1 ml in all instances. At the lower levels of vitamin A supplementation the vitamin was fed in individual fraction cups, but with the larger dosages (1,760 U.S.P. units daily and above) the supplement was placed directly in the rat's stomach by means of a special needle attached to a tuberculin syringe. The feeding of the supplement was continued for 28 days. The animals were then slaughtered and their livers removed, saponified and their vitamin A content determined in the manner previously described. The vitamin A content of the various livers was calculated in terms of the new U.S.P. standard and the values reported are average values for all animals constituting the respective experimental groups.

During the course of the previously described experiments other nutritional studies involving various diets were in progress in the laboratory. It was believed that a study of hepatic vitamin A storage under these experimental conditions would be of interest. Therefore, at the termination of the experiments the animals were sacrificed and the vitamin A content of their livers determined. Among the animals investigated were those which had previously received the Steenbock rachitogenic diet, the U.S.P. thiamine deficient diet, a breakfast-cereal diet, and the latter diet supplemented with milk, with vegetables, and with both milk and vegetables.

The pertinent data obtained in the course of these studies have been condensed and arranged in tabular form and are presented in tables 1 to 3, inclusive.

## DISCUSSION

In the present studies it was of interest to find that the liver of the newborn rat contains only a limited amount of vitamin A (see table 1) and that the storage of the vitamin in that tissue did not increase greatly during the nursing period.

TABLE 1

*Vitamin A content of livers of rats of various ages taken directly from the breeding colony*

| GROUP NO. | NO. OF RATS USED | SEX | AGE OF RATS | WEIGHT OF RATS (ave.) | WEIGHT OF LIVERS (ave.) | TOTAL UNITS FOUND (ave.) | UNITS/GM LIVER (ave.) | UNITS/GM BODY WEIGHT (ave.) |
|---|---|---|---|---|---|---|---|---|
| | | | *days* | *gm* | *gm* | *U.S.P.* | *U.S.P.* | *U.S.P.* |
| 1 | 8 | ? | 1 | 6.2 | 0.36 | 7 | 20 | 1.1 |
| 2 | 5 | ? | 7 | 11.2 | 0.70 | 25 | 44 | 3.9 |
| 3 | 5 | Males | 16 | 30 | 1.98 | 105 | 54 | 3.5 |
| 4 | 4 | Males | 21 | 42 | 2.6 | 130 | 50 | 3.1 |
| 5 | 4 | Males | 37 | 89 | 5.9 | 360 | 61 | 4.0 |
| 6 | 4 | Males | 51 | 127 | 7.3 | 700 | 95 | 5.5 |
| 7 | 3 | Males | 73 | 212 | 10.1 | 1,860 | 184 | 8.8 |
| 8 | 1 | Female | 117 | 178 | 8.7 | 5,700 | 655 | 32.0 |
| 9 | 6 | Males | 170 | 361 | 12.2 | 22,470 | 1,862 | 63.0 |
| 10 | 3 | Females | 330 | 241 | 10.2 | 18,950 | 1,769 | 82.3 |
| 11 | 1 | Female | 334 | 202 | 9.5 | 16,190 | 1,704 | 77.8 |
| 12 | 1 | Female | 437 | 279 | 10.4 | 19,840 | 1,908 | 71.3 |
| 13 | 4 | Females | 564 | 281 | 10.4 | 18,440 | 1,762 | 65.9 |
| 14 | 4 | Females | 741 | 269 | 10.9 | 18,600 | 1,708 | 69.5 |
| 15 | 6 | Males | 749 | 421 | 14.5 | 28,400 | 1,865 | 68.5 |

This seems to indicate that the vitamin A present in the dam's milk is barely enough to provide for the active physiological processes associated with the rapid growth of the young. That the rate of metabolism is a factor in vitamin A storage in the rat is indicated by the fact that as a rule those weanling rats which consume the most food and which grow most rapidly during the early days of the vitamin A depletion period are invariably the animals which manifest deficiency symptoms

at the earliest date. However, the recent studies of Johnson and Baumann ('48) indicate that a 50% increase in growth rate is more conducive to low liver storage of vitamin A than is a three-fold increase in metabolic rate induced by feeding desiccated thyroid.

Livers of day-old rats were found to contain an average of 7 U.S.P. units of vitamin A per liver, or 20 units per gram of liver. The former value is in agreement with the value reported by Williamson ('48) for the vitamin A content of livers of normal unborn rats and hence may be indicative of the amount of vitamin A imparted to the young through placental circulation.

The livers from the 7-day-old rats appeared to have undergone a three-fold increase in their vitamin A content during the first week of postnatal life. However, owing to an increase in liver size the increase in vitamin A concentration was only two-fold during this period. The livers from other young rats slaughtered at 16 and 21 days of age, respectively, showed further increases in vitamin A deposition, but the increase in vitamin concentration per gram of rat liver or per gram of body weight during this period appeared rather insignificant. Perhaps this again reflects the vitamin content of rat's milk; it may indicate why the nursing young are readily depleted of their body stores of vitamin A. Livers from 37-day-old rats were found to contain almost three times as much vitamin A as livers from 21-day-old animals, and this increase in vitamin store was reflected in greater concentration of the vitamin per unit weight of liver and per gram of body weight. As the age of the rats increased further, the amount and the concentration of vitamin A found in the liver also increased rapidly. Livers from 51-day-old rats were found to contain twice as much vitamin A as did livers from 37-day-old rats, while livers from 73-day-old rats contained two and one-half times as much vitamin A as did livers from 51-day-old rats. This rapid increase in hepatic vitamin A with increase in age would seem to explain why it is extremely difficult to deplete the older animals.

Hepatic vitamin A continued to increase with age. The livers from 170-day-old male rats were found to contain more than 10 times as much of the vitamin as did the livers from 73-day-old rats, whereas the livers from 749-day-old males contained more than 15 times as much. Similarly, the hepatic vitamin A of female rats which had previously weaned litters and then undergone a 20-day rest period was found to increase so long as the body weight increased. The liver of a 117-day-old female weighing 178 gm was found to contain 5,700 U.S.P. units of vitamin A, whereas the livers from 330-day-old females (average weight, 241 gm) contained 18,950 units. However, further increases in the age of the females without significant increases in body weight did not result in any appreciable increase in liver storage of the vitamin. It was of particular interest to find that the hepatic vitamin A of both male and female rats reached its maximum concentration when the rats were approximately 170 days old. Although vitamin A storage increased beyond this age, the concentration in the liver did not increase. Since hepatic vitamin A values considerably greater than those found in the present studies have been observed, it is believed that the values herein reported do not represent saturation values but instead are probably optimum storage values for the dietary regime employed.

Livers from young rats which had been subjected to vitamin A depletion to the extent that they lost about 20% of their maximum body weight were found to contain no vitamin when the concentrates were examined by the antimony trichloride method (table 2). On the other hand, when the nonsaponifiable residues from similar livers were dissolved in isopropyl alcohol and the solutions examined at 328 mμ by means of the Beckman quartz spectrophotometer, a small but definite amount of absorption was invariably indicated. It was believed that this absorption was due to extraneous materials present in the nonsaponified fraction and not to vitamin A. However, spectrographic examination of concentrates from other rat livers known to contain vitamin A indicated a

TABLE 2

*Relation of the vitamin A intake of the rat to the amount stored in the liver while subsisting on the U.S.P. vitamin A-deficient diet*

| ANIMAL GROUP NO.[1] | UNITS FED DAILY [2] | CHANGE IN WEIGHT [3] (AVE.) | TOTAL UNITS FOUND (AVE.) | UNITS/GM LIVER (AVE.) | UNITS/GM BODY WEIGHT (AVE.) | VITAMIN A RECOVERED (AVE.) |
|---|---|---|---|---|---|---|
| | *U.S.P.* | *gm.* | *U.S.P.* | *U.S.P.* | *U.S.P.* | *%* |
| 1 | None [4] | .... | 0 | 0.0 | 0.00 | .. |
| 2 | 0.73 | + 12 | 18 | 3.9 | 0.12 | 88 |
| 3 | 2.2 | + 67 | 34 | 4.1 | 0.17 | 55 |
| 3A [5] | 2.2 | + 64 | 37 | 4.2 | 0.19 | 60 |
| 4 | 4.4 | + 69 | 52 | 5.1 | 0.26 | 42 |
| 5 | 7.4 | + 70 | 59 | 5.7 | 0.31 | 28 |
| 6 | 14.7 | + 74 | 61 | 5.7 | 0.31 | 15 |
| 7 | 22 | + 79 | 68 | 6.1 | 0.33 | 11 |
| 8 | 29 | + 80 | 71 | 6.5 | 0.34 | 9 |
| 9 | 44 | + 98 | 160 | 12.8 | 0.70 | 13 |
| 10 | 88 | + 85 | 340 | 28.8 | 1.62 | 14 |
| 11 | 176 | + 87 | 550 | 44.8 | 2.57 | 11 |
| 12 | 290 | + 76 | 1,070 | 87.7 | 4.8 | 13 |
| 13 | 590 | + 82 | 2,930 | 210 | 12.9 | 18 |
| 14 | 880 | + 76 | 3,900 | 290 | 17.6 | 16 |
| 15 | 1,760 | + 90 | 8,950 | 640 | 40.3 | 19 |
| 16 | 2,640 | + 77 | 9,360 | 730 | 45.2 | 13 |
| 17 | 3,520 | + 85 | 13,690 | 1,140 | 62.5 | 14 |
| 18 | 4,400 | + 72 | 62,820 | 5,510 | 310 | 51 |
| 19 | 6,600 | + 65 | 74,100 | 6,330 | 380 | 40 |
| 20 | 8,800 | + 71 | 92,580 | 8,050 | 450 | 38 |
| 21 | 17,600 | + 20 | 105,360 | 12,630 | 700 | 21 |
| 22 | 18,380 | + 20 | 92,210 | 9,510 | 630 | 18 |
| 23 | 26,400 | + 8 | 117,260 | 11,380 | 860 | 16 |
| 24 | 35,200 | — 22 | 116,800 | 13,710 | 1,050 | 12 |
| 25 | 37,760 | — 27 | 87,330 | 11,640 | 960 | 6 |
| 26 | 75,520 | — 21 | 104,990 | 12,780 | 970 | 5 |

[1] Consisting of from 5 to 12 rats per group.

[2] Fed for a 28-day test period.

[3] Average weight of rats at end of depletion period was 128 gm.

[4] The animals of this group were maintained on the vitamin A-deficient diet for approximately 32 days or until they had lost, on an average, 28.4% of their maximum weight.

[5] The animals of this group were fed 3 mg of alpha-tocopherol daily during the 28-day test period as an additional supplement.

somewhat higher vitamin potency than did the results of the antimony trichloride method of assay.

When depleted rats were fed increasing doses of vitamin A ranging from 0.73 to 29 U.S.P. units per day over a 28-day period, there was a slight but measurable increase in the total amount of vitamin A stored in the livers, and the efficiency of storage, at the lower levels of intake, was found to be relatively high. On increasing the dosage to 44 U.S.P. units daily, the rats attained a maximum rate of growth and showed uniformly marked increases in hepatic vitamin A, as indicated both by total units and by concentrations. Further increasing the daily vitamin A intake in progressive stages from 44 through 8,800 U.S.P. units resulted in greatly increased amounts of hepatic vitamin A with each increment in intake. In fact, when the daily dosage ranged from 29 to 3,520 U.S.P. units, the hepatic vitamin A was found to approximately double for each two-fold increase in daily intake. However, over this range of vitamin intake the rates of growth of the animals constituting the several experimental groups were approximately the same. On increasing the vitamin A dosage from 17,600 to 75,520 U.S.P. units daily, in progressive amounts, there was no appreciable increase in hepatic vitamin A. In fact, the data indicate that somewhat less vitamin A was stored on the highest levels of intake (37,760 and 75,520 U.S.P. units daily) than on some of the lower levels of intake (26,400 and 35,200 U.S.P. units daily), but this condition is believed to be related to the depressed growth rate associated with the higher levels of vitamin intake. However, with these higher rates of intake the concentrations of vitamin A per gram of liver or per gram of body weight were in line with those obtained on the lower levels of intake. In connection with these storage studies it was of interest to observe that a previously depleted female rat, when fed 8,800 U.S.P. units of vitamin A daily for 28 days, retained sufficient vitamin to permit the production of three litters of young and the weaning of two litters, and to prolong the life of the female for 492 days.

In these studies it was noted that no improvement in growth rate resulted from feeding daily dosages of vitamin A in excess of 44 U.S.P. units, although the concentration of vitamin in the liver was far from optimum at that level of intake. Furthermore, it was noted that when the daily vitamin A intake was increased beyond 8,800 U.S.P. units daily, the growth rate was rather consistently depressed. The physiological complications arising from excessive intakes of vitamin A merit study and, as previously mentioned, the matter is already receiving consideration in this laboratory. The amount of vitamin A required to produce optimum growth in the present studies (44 U.S.P. units daily) is somewhat higher than that reported by Lewis et al. ('42); the difference may be due to the fact that the animals used in the present studies were depleted before being placed on test.

It has frequently been suggested that the vitamin E intake has an important bearing on vitamin A storage (Moore, '31; Davies and Moore, '41; and others). To determine whether hepatic vitamin A could be increased under the conditions prevailing in the present experiments by feeding a vitamin E supplement, a group of 11 depleted rats (Group 3A) was fed 3 mg alpha-tocopherol daily, along with 2.2 U.S.P. units of vitamin A, during the usual 28-day feeding period. The results show that there was no increase in either the growth rate or in the amount of vitamin A stored as the result of feeding the tocopherol supplement.

Another factor which appears to influence vitamin A storage is the duration of the period of vitamin A feeding. Glover et al. ('47) found that when moderate to extremely high dosages of vitamin A were administered in the form of a single dose to 6- to 9-month-old-rats and their livers examined from three to 14 days afterwards, rather inefficient liver storage of the vitamin had resulted (1 to 7%). On the other hand, Lemley et al. ('47), using a three-day feeding period, reported a more efficient storage of vitamin A than was observed in the present investigation except where the dosage ranged from 4,400 to 8,800 U.S.P. units daily. In this connection, it was of

interest to note that the most efficient hepatic vitamin A storage occurred when the vitamin A intake was approximately 4,000 units daily.

Foy and Morgareidge ('48), using a modification of the Guggenheim and Koch technic ('44), found that when partially depleted rats were fed daily doses of vitamin A ranging from 71 to 298 U.S.P. units for two successive days, and then slaughtered on the 4th day, from 13 to 38% of the vitamin could be recovered from the livers. According to the data of these authors, the amount of the vitamin stored in the liver of the rat depended on the intake, and the efficiency of the storage seemed to be greatest where the higher dosage was fed. On the other hand, according to our data, if somewhat comparable amounts of vitamin A (142 and 586 U.S.P. units) had been fed to fully depleted rats over a 28-day period, the efficiency of vitamin storage would have been greatest with the lower level of intake. Our findings show that the efficiency of vitamin A storage was lowest where the dosage was in the range of the minimum amount required to produce optimum growth, or where extremely high doses were fed. In these respects the data herein reported confirm those previously reported by Lemley et al. ('47). The present data are also in agreement with those of Lewis et al. ('41) in that maximum growth in the rat was obtained with a daily vitamin A intake ranging from 25 to 50 U.S.P. units, but the data do not confirm the findings of these authors that more than 10 units of vitamin A must be fed daily before any vitamin is stored in the liver.

An examination of the results obtained by assaying the livers of rats which had been maintained on several experimental diets for specific periods reveals some points of interest (table 3). Livers from 52-day-old rats which had subsisted on the Steenbock rachitogenic diet for 28 days contained approximately one-third as much vitamin A as did the livers from rats of comparable age taken directly from the breeding colony. Since the rats which had received the rachitogenic diet were subnormal in weight, part of this difference is accounted for by the difference in weight of the

TABLE 3

*Concentration of vitamin A [1] in liver of rats which had subsisted on various experimental diets*

| GROUP NO. | TYPE OF DIET FED | NUMBER OF RATS USED | FINAL AGE | DAYS ON EXPERIMENT | FINAL WEIGHT (AVERAGE) | WEIGHT OF LIVER (AVERAGE) | TOTAL UNITS FOUND (AVERAGE) | UNITS/GM LIVER (AVERAGE) | UNITS/GM BODY WEIGHT (AVERAGE) |
|---|---|---|---|---|---|---|---|---|---|
| | | | *days* | | *gm* | *gm* | *U.S.P.* | *U.S.P.* | *U.S.P.* |
| 1 | Steenbock's rachitogenic | 6 | 52 | 28 | 71 | 5.2 | 220 | 41 | 3.0 |
| 2 | Mixed cereals (breakfast foods) | 6 | 64 | 42 | 78 | 5.4 | 90 | 17 | 1.1 |
| 3 | Mixed cereals + milk (10 ml daily) | 6 | 64 | 42 | 176 | 9.1 | 400 | 44 | 2.3 |
| 4 | Mixed cereals + vegetables | 6 | 64 | 42 | 86 | 5.8 | 1,290 | 224 | 15.0 |
| 5 | Mixed cereals + milk and vegetables | 6 | 64 | 42 | 167 | 9.2 | 2,200 | 240 | 13.2 |
| 6 | Purified ration containing 2% cod liver oil | 10 | 222 | 196 | 183 | 9.0 | 4,830 | 544 | 26.4 |
| 7 | Purified ration containing 2% cod liver oil | 10 | 262 | 238 | 209 | 9.7 | 7,180 | 754 | 34.4 |

[1] Exclusive of carotene.

storage organ. Based upon the findings of Lewis et al. ('42) that liver storage of vitamin A in the range of 34 units per gram of tissue is associated with normal plasma vitamin A, the present data indicate that the Steenbock rachitogenic diet is fully adequate with respect to this vitamin. On the other hand, livers from 64-day-old rats which had subsisted for 42 days on a diet composed of a mixture of breakfast cereals contained approximately one-twentieth of the amount, or one-fourth of the concentration, of vitamin A found in the livers of normal rats of the same age. When the cereal diet was supplemented by supplying the test animal with 10 ml of fresh milk daily, the liver vitamin A increased 4-fold, while the concentration of the vitamin in the liver more than doubled. On the other hand, when the cereal diet was supplemented with a liberal allowance of canned vegetables daily (peas, carrots, tomatoes, etc.), the hepatic vitamin A increased over 14-fold, while the concentration of the vitamin in the liver tissue increased to the same extent. However, growth was subnormal due to deficiencies other than vitamin A. When both the milk and the vegetable supplement was fed, a further increase in vitamin A storage resulted. Since the milk supplement resulted in an accelerated growth rate, the concentration of the vitamin in the liver tissue did not increase proportionally, owing to the increase in liver tissue.

Purified diets containing 2% of cod liver oil, such as have been widely used in B-vitamin investigations, apparently furnish ample amounts of vitamin A even when fed over extensive periods.

## SUMMARY

A study was made of the effects of age and of the vitamin A intake of the rat on vitamin A storage as indicated by the vitamin A content of the liver, this content being determined on the nonsaponifiable extract of the liver by means of the antimony trichloride method. The results obtained during the course of this study were as follows:

1. The liver of the normal newly-born rat contained very little vitamin A.

2. The concentration of vitamin A in the liver of the nursing rat more than doubled during the first 7 days of postnatal life and then remained relatively constant during the remainder of the nursing period.

3. The amount and the concentration of vitamin A in the liver of the normal weanling rat increased rapidly throughout the period of rapid growth and reached a maximum concentration when the rat was about 170 days old. Beyond this age, additional vitamin A was stored but the concentration per gram of liver remained relatively constant.

4. The amount of vitamin A stored in the livers of depleted rats depended upon the intake, but no marked increase in concentration of hepatic vitamin A occurred until the intake was more than sufficient to promote optimum growth. Below this level of intake, increase in vitamin A storage as indicated by increased concentration was largely hidden by increase in weight of liver.

5. With vitamin A intakes in excess of that required for optimum growth, liver storage increased in proportion to the intake until a daily intake of 17,600 U.S.P. units was reached. Intakes of vitamin A in excess of this amount did not result consistently in additional vitamin A storage when fed under the conditions of these experiments.

6. Optimum growth was brought about in depleted rats when 44 U.S.P. units of vitamin A were fed daily, whereas daily dosages in excess of 8,800 U.S.P. units had a definite depressive effect on the growth rate.

The effects on liver storage of vitamin A in the rat resulting from ingestion of other types of experimental diets are presented and discussed.

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# THE THIAMINE REQUIREMENT OF THE NORMAL INFANT [1]

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TWELVE FIGURES

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The problem of assessing vitamin requirements has been complicated by the discovery of a number of variable factors which may influence them. Vitamin requirements are conditioned by pathological processes which affect absorption or utilization or increase the need for particular factors. But even in the normal subject there are wide fluctuations in requirements, caused by the nature of the food, its effect on the bacterial synthesis of vitamins in the intestine, and its content of antivitamins. Alterations in the diet and bacteriostatic drugs are known to affect the synthesis of vitamins in the intestine. Vitamins present in the diet may be destroyed before ingestion by exposure, processing or cooking, and in the intestine they may be rendered unavailable by agents which compete with, combine with, adsorb or destroy them.

In the case of the infant many of these complicating factors do not enter the picture. The infant artificially fed along conventional lines receives a diet which is relatively constant in its proportion of calorigenic foodstuffs and in their source,

[1] The expenses of this study were defrayed in part by grants from the American Dairy Association, the Sugar Research Foundation, and Mead Johnson and Company.

and antivitamins are not known to be present. Under these conditions it would seem possible to define vitamin requirements with reasonable accuracy and to determine what margin of safety our usual artificial feedings provide. The present report deals with an attempt to determine the minimum requirement of the normal infant for thiamine.

A minimum food requirement may be defined as that intake which will just protect against definite clinical symptoms or accepted laboratory criteria of deficiency. It was not deemed justifiable to deplete infants to the point of clinical deficiency, and laboratory criteria were therefore used as an endpoint. The selection of such criteria deserves some discussion since there is lack of agreement in regard to their merits. Thiamine deficiency has been measured by assays of thiamine in biopsied tissues, by determination of thiamine in serum, by the cocarboxylase level of the blood cells, by the pyruvate levels or the pyruvate: lactate ratio of the blood serum, and by various excretion procedures for measuring urinary thiamine. The biopsy technique, aside from obvious objections, is undesirable because of its inaccuracy and the paucity of "normal" standards. Pyruvate levels and pyruvate:lactate ratios are influenced by non-specific factors (exercise, excitement) which are difficult to control in infants and were therefore to be avoided. The analytical methods for blood thiamine and cocarboxylase measurement at the time our study was begun were not considered sufficiently reliable and accurate, and some urinary excretion procedure therefore seemed the best method for estimating the degree of deficiency.

Three types of urinary excretion tests are commonly employed: (1) the 24-hour urinary excretion test, (2) load tests, and (3) fasting excretion tests. These have been applied to thiamine itself and to its pyrimidine decomposition product variously known as "pyramine" or PAYF (pyrimidine activator of yeast fermentation), an inert metabolite. We employed the 24-hour excretion test as being the most suitable for our purpose and confined our observations to the

excretion of thiamine itself. Although a load test has certain advantages, particularly in evaluating well-developed clinical deficiencies, we did not use one because of the delay involved in reaching equilibrium after the introduction of a test dose. Ordinarily the 24-hour excretion test is objectionable in that a postprandial rise in excretion follows the ingestion of a thiamine-containing meal, regardless of whether a subject is deficient or not. We have found, however, that on low thiamine intakes, and when the thiamine is administered in divided doses, the rise in excretion does not occur, the output remaining relatively constant throughout the 24 hours at a level approximating the fasting excretion level. The 24-hour urine obtained under these conditions therefore yields the same information as the fasting excretion test and obviates the necessity of accurate timing of samples for collection; furthermore it provides larger quantities of material to work with.

Since Mickelsen, Castor and Keys ('47) have stressed the advantages of measuring the "pyramine" rather than the thiamine excretion, it may be in order to point out why we preferred the latter. Although these authors called attention to the greater individual variations in thiamine than in "pyramine" excretion, this was not true of the low range bordering on deficiency with which we were particularly concerned. An even more important consideration is the fact that the thiamine excretion curve, unlike the "pyramine" curve, shows a sharp break which makes a convenient, and we believe a significant, point of reference. Unlike "pyramine" excretion, which decreases in a continuous exponential curve as the intake is reduced, thiamine excretion decreases as a linear function of the intake until a certain critical point is reached which we have termed the "point of minimum excretion." Decreasing the intake beyond this point causes only a minimum further decrease in thiamine excretion. We have observed this phenomenon in all adults and infants we have studied. Evidence of it may also be found in the observations of Knott ('40) and of Mason and Williams ('42). Our inter-

pretation of this phenomenon, which is shared by Mason and Williams, is that the higher excretion rate above this critical point represents a surplus above minimum physiological needs. This hypothesis was tested experimentally by Najjar and Holt ('43)[2] in adult subjects who were carried to the point of thiamine deficiency symptoms. The point of minimum urinary excretion was attained when the intake was reduced to 0.6 mg per day; when the intake was gradually reduced beyond this point during a period of many months, frank deficiency symptoms were noted at an intake of 0.2 mg per day. Since undetected tissue alterations may well have occurred some time before the appearance of the frank symptoms, it seems probable that the beginning of deficiency is associated with an intake not far below that which gives rise to "minimum excretion." Further support for this point of view was obtained by Salcedo et al. ('48) in experiments on thiamine-depleted rats in which the concentration of thiamine in various tissues was correlated with the development of minimum urinary excretion. The course of depletion was associated with a progressive decrease in thiamine concentration in the liver, kidney and heart; the brain, on the other hand, maintained its concentration of thiamine until the point of minimum excretion was reached, after which the thiamine content fell abruptly.

Since the data referred to above indicated that the "point of minimum excretion" was apparently close to, if not identical with, the beginning of subclinical deficiency and that it could be attained with reasonable safety and without risk of clinical deficiency symptoms, we undertook to determine this point with accuracy in a group of infants as a measure of the minimum requirement for thiamine.

## EXPERIMENTAL

### *Subjects*

The subjects of this study were 7 healthy male infants. Five ranged between 7 and 10 months of age, weighing between

[2] See also Holt ('44).

TABLE 1

*Composition of the salt mixture* [1]

| INGREDIENT | QUANTITY | INGREDIENT | QUANTITY |
|---|---|---|---|
| | % | | % |
| NaCl | 18.9 | Fe citrate | 2.21 |
| $CaH_2PO_4$ | 25.0 | $CuSO_4$ | 0.24 |
| $MgSO_4$ | 6.8 | $MnSO_4$ | 0.15 |
| $KHCO_3$ | 44.4 | KI | 0.015 |
| KCl | 2.88 | NaF | 0.03 |

[1] Incorporated as 5 gm of mixture per 750 Cal. of diet.

5.9 and 7.7 kg at the start; the other two were one month and three months of age and weighed 4.1 and 4.7 kg respectively. They were placed on metabolism frames for a 5-day period each week during which 24-hour urine specimens were collected; this was followed by a two-day rest period.

### *Diet*

In order to secure a basal diet essentially free of vitamins, to which vitamins could be added in accurately weighed quantities, a purified diet was employed consisting of vitamin-free casein, vitamin-free dextrimaltose,[3] hydrogenated cottonseed oil,[4] and a salt mixture (table 1); these constituents were mixed with water to provide a feeding, cereal-like in consistency. The diet was so composed that 50% of the calories were supplied by the carbohydrate, 35% by the fat and 15% by the protein. One hundred calories per kilogram of body weight were fed daily. All the vitamins were administered as supplements. Vitamins A and D were given in the form of oleum percomorphum, 15 drops daily. The water-soluble vitamins (table 2) were divided into 4 equal doses and administered midway between meals; this was done to avoid the spilling that occurs in the urine as a result of a single large dose.

[3] Specially prepared by Mead Johnson & Company through the courtesy of Dr. Warren M. Cox, Jr.

[4] Crisco. Contributed by Procter and Gamble.

### *Laboratory methods*

Urine was collected in dark bottles containing sufficient glacial acetic acid to make a 2% solution. The thiamine content of each 24-hour specimen was determined fluorimetrically

TABLE 2

*Composition of vitamin mixture (daily dose)*

| VITAMIN | QUANTITY | VITAMIN | QUANTITY |
|---|---|---|---|
| | *mg* | | *mg* |
| Thiamine chloride | varied | Para-aminobenzoic acid | 0.167 |
| Riboflavin | 0.167 | Choline | 0.833 |
| Nicotinamide | 4.16 | Inositol | 0.167 |
| Pyridoxine | 0.167 | Ascorbic acid | 4.0–12.0 [1] |
| Calcium pantothenate | 0.167 | | |

[1]The small dose (4.0 mg per day) was given to three infants in this study. It was calculated to provide an adequate requirement on the basis of an adult study (Najjar et al., '44) in which it had been shown that 25 mg per day would protect a 70 kg adult, the assumption being that the infant's requirement would be proportional to his weight. This intake proved adequate for two infants but inadequate for a third, who suffered from repeated attacks of otitis media and who developed clinical scurvy 6 weeks after his first illness and 5 months after he had been placed on this regimen. This precipitation of scurvy in the face of infection is in accord with the findings of Hamil et al. ('38), who found that 10 mg was adequate during health but failed to give full protection during periods of infection. After this incident the ascorbic acid intake was raised to 12 mg.

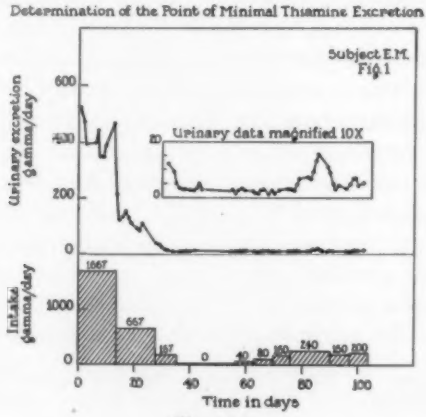


Figure 1

by the thiochrome procedure of Najjar and Ketron ('44). It should be emphasized that at these low levels of thiamine excretion the introduction of a correction factor for the presence of fluorescent derivatives of nicotinic acid, as is done in the Najjar-Ketron method, becomes a matter of great importance if accurate measurements of thiamine are to be obtained.

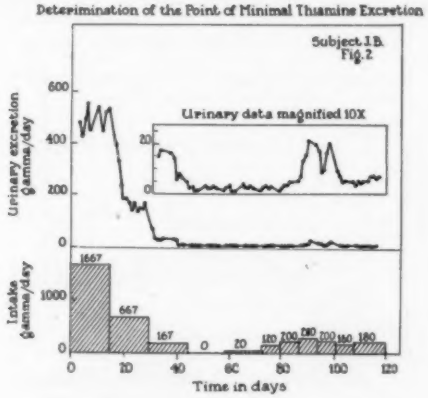


Figure 2

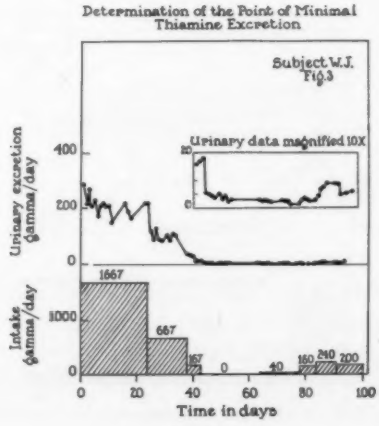


Figure 3

### *Plan of experiment*

In order to ascertain the "point of minimum excretion" of thiamine as quickly as possible, the following procedure was followed. After two periods on a high thiamine intake (1667 and 667 μg per day) the thiamine was entirely omitted for a period of 14 to 21 days to permit the urinary excretion to become stabilized at the minimum level. The periods of high

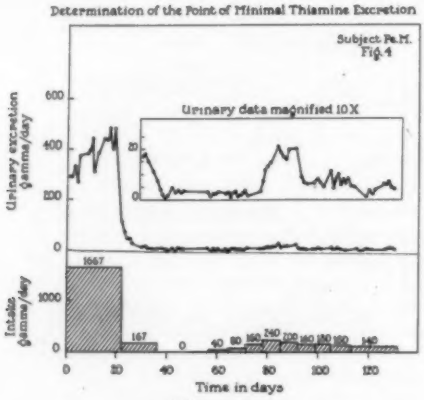


Figure 4

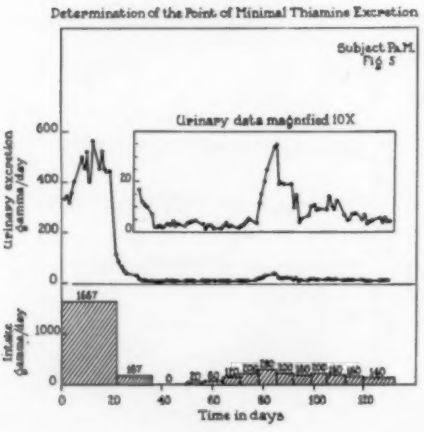


Figure 5

intake were omitted in the last two subjects; they were started immediately on a period of zero thiamine intake. Thiamine was then added cautiously in small amounts, increasing the intake at intervals of a week or two until excretion beyond the minimum occurred. The thiamine intake was then adjusted by subsequent small decreases and increases until an intake was obtained which would maintain excretion just above the minimum point.

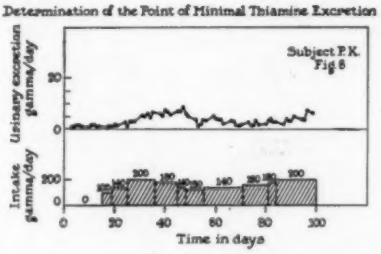


Figure 6

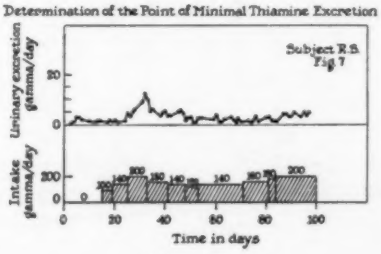


Figure 7

## RESULTS

Protocols of the 7 subjects studied are shown graphically in figures 1–7. It is apparent that the minimum excretion in these infants corresponds to a 24-hour output of 1 to 5 μg per day. There appeared to be a slight variation among subjects; the smaller figures were more regularly encountered in the smaller subjects. A similar variation was found by Najjar

and Holt ('43) in adults, in whom minimum excretions of between 36 to 60 μg per day (1.5 to 2.5 μg per fasting hour) were observed.

The intake which will produce an excretion just in excess of the minimum level proved to be singularly constant in these infants, being 0.14 mg in one subject, 0.16 in two subjects, and 0.20 in the remaining 4. Reduction of intake below these amounts invariably resulted in an output of less than

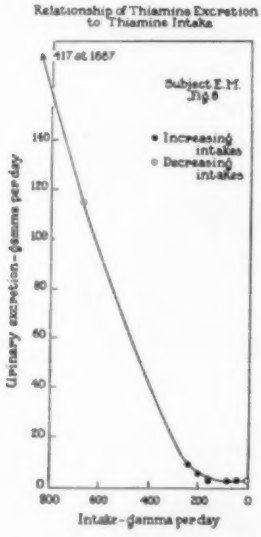


Figure 8

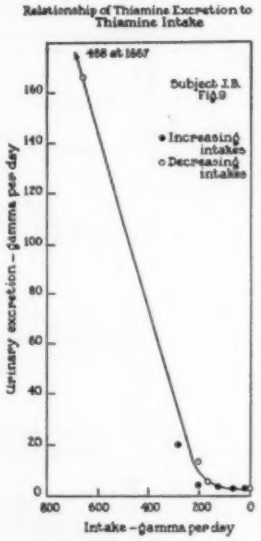


Figure 9

5 μg per day in the urine, whereas these quantities would maintain an output of between 5 and 10 μg per day. Any further increase produced a sharp rise in thiamine excretion.

The relation between intake and excretion is shown graphically in figures 8–12. The linear relationship between urinary excretion and intake is clearly seen with intakes above the critical point. Roughly one quarter of the added thiamine is excreted as such in the urine, a proportion slightly greater than that reported for adults in the extensive studies of

Mickelson et al. ('47), although, as we have mentioned, considerable variations were encountered in their study. The relation between thiamine excretion and intake in our infants appears to be far more constant at higher levels of intake than that observed by the Minnesota workers in their adult subjects.

Despite the fact that our subjects were maintained for 4 to 5 months on a low thiamine intake close to what we have

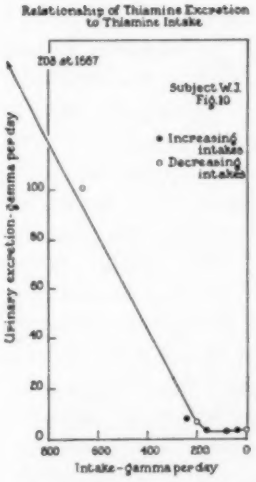


Figure 10

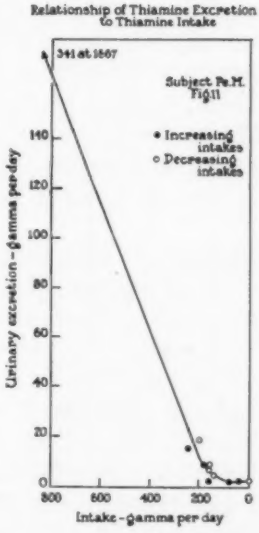


Figure 11

regarded as the minimum, no signs or symptoms of thiamine deficiency developed. As far as could be ascertained by daily physical examinations with special search for neurological abnormalities, careful observation of the infants' behavior, and laboratory data such as complete blood counts, urinalyses, x-rays of the long bones, and electrocardiograms, these infants continued to progress in an essentially normal fashion. However, after approximately 6 months on the purified diet,

we quite regularly encountered a diminution or cessation of gain in weight. This is not related to thiamine lack, since prolonged administration of large amounts of thiamine at this point does not alter the weight curve. Further evidence that thiamine deficiency was not concerned in this failure to gain weight was furnished by unpublished experiments in which similar behavior was noted when the same purified

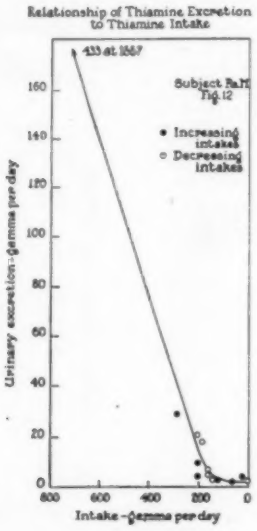


Figure 12

diet was fed without any thiamine restriction. This disturbance must be attributed to some other factor deficient in the synthetic diet; we have noted that it could be corrected by brewers' yeast in the majority of cases in which this was tried. In the two subjects (P. K. and R. S.) in whom it was possible to attain the endpoint rapidly, weight gain proceeded at a very satisfactory rate despite the restriction of the thiamine intake to from 0.14 to 0.20 mg daily for a considerable period of time.

## COMMENT

It is of interest to compare the minimum thiamine requirement of 0.14 to 0.20 mg per day, as determined by this technique, with the intake which the average artificially-fed infant receives. Based on an average content of 35 to 40 μg of thiamine per 100 ml of cow's milk, an infant weighing 7 kg would receive at least 300 μg a day, apparently an ample margin of safety beyond a daily requirement of 200 μg. On the other hand, the destruction of thiamine by heat in the pasteurization and sterilization of milk formulas must be considered. According to Kon ('47), 10% of the thiamine may be destroyed by the former process and 30% or even more by more drastic sterilization procedures. The margin of safety with sterilized milk would thus appear to be very small indeed, and the possibility that autoclaving milk, which is becoming increasingly popular, may destroy even more thiamine is one that demands careful study.

Since breast milk contains roughly half as much thiamine as cow's milk, the margin of safety it provides would seem to be decidedly small. We hesitate to draw such a conclusion from present data, however, since there is reason for believing that some thiamine is formed from intestinal bacteria and it is possible that the flora of the breast-fed infant provides more of a supplement than that of the infant on a cow's milk diet. The data in the literature are inadequate to settle this point. A purified diet simulating more closely the composition of breast milk should throw light on this question. It should be emphasized that the figures in our study are applicable only to the conditions prevailing therein.

## SUMMARY

The thiamine requirement of normal infants was determined by means of a urinary excretion procedure which involved determining the intake that would maintain urinary excretion at the upper limit of the zone of minimum excretion. The requirement varied between 0.14 mg and 0.20 mg per day in the 7 subjects studied.

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# VITAMIN STUDIES IN MIDDLE-AGED AND OLD INDIVIDUALS

## II. CORRELATION BETWEEN VITAMIN A PLASMA CONTENT AND CERTAIN CLINICAL AND LARORATORY FINDINGS [1]

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FIVE FIGURES

(Received for publication August 26, 1948)

Although the literature dealing with the clinical aspects of hypovitaminosis A is quite extensive, few reports are available on the correlation between vitamin A plasma values and the objective symptoms presented by individuals. The present study was undertaken with the purpose of supplying such data.

The study included estimation of the frequency of occurrence of such signs as are generally accepted as being associated with a condition of insufficient intake of vitamin A, e.g., impaired dark adaptation, xerosis of the conjunctiva, Bitot's spots, blepharoconjunctivitis, dryness and scaliness of the skin, and follicular hyperkeratosis (toad skin). In addition to these clinical observations, studies were undertaken with the purpose of evaluating the degree of epithelial damage. The procedures employed included a determination of the daily excretion of epithelial cells in the urine and an examination of the percentage of keratinized epithelial cells in the urinary sediment. Furthermore, smears were made from the conjunctiva of both eyes and the percentage of cornified cells present was calculated from a differential count of the stained smears.

[1] Funds provided by Hoffmann-La Roche, Inc., Nutley, New Jersey.

The technique of plasma vitamin A estimation used and the values observed in a large group of individuals of different ages have been reported in a previous publication (Kirk and Chieffi, '48). The subjects included in the present investigation were chosen from the same group of inmates and patients in the St. Louis City Infirmary and Infirmary Hospital.

## EXPERIMENTAL

Examinations for xerosis of the conjunctiva, localized conjunctival thickening, blepharoconjunctivitis, dryness and scaliness of the skin and toad skin were made on 106 subjects.

The examination for *xerosis* was performed by everting the lower eyelid and exposing the palpebral and bulbar conjunctiva to the air for two minutes. In no instance was dryness of the conjunctival mucous membrane noted after this period of exposure.

The percentage frequency of the occurrence of the other symptoms is listed in table 1, which also contains the calculated correlation significance both with regard to the plasma vitamin A concentration and to age. The various findings for each symptom will be discussed below.

### *Localized thickening of the conjunctiva*

The conjunctival changes characteristic of vitamin A hypovitaminosis have, in the last few years, been the subject of much discussion. The conjunctival spots, as first described by Bitot in 1863, in patients suffering from poor dark vision were located just lateral to the cornea in the horizontal meridian of the eye, generally being in the shape of a triangle the base of which was slightly concave and about 5 mm in width, while the sides of the triangle were about 8 mm in length; the spots were usually covered by a white or yellow foamlike substance, presumably consisting of metaplastic epithelial cells. In an investigation by Kruse ('41), such Bitot "spots" were claimed to have been observed in a large percentage of the general population. The identification of

TABLE 1

*Correlation of various clinical signs in 106 individuals with vitamin A concentration of plasma and with age*

| SYMPTOMS | Plasma vitamin A in µg% 1–15 / Age in years 16–99 / Number of persons 61 | Plasma vitamin A in µg% 25–60 / Age in years 16–99 / Number of persons 45 | "t" test | Plasma vitamin A in µg% 1–15 / Age in years 16–59 / Number of persons 18 | Plasma vitamin A in µg% 25–60 / Age in years 16–59 / Number of persons 21 | "t" test | Plasma vitamin A in µg% 1–15 / Age in years 60–99 / Number of persons 43 | Plasma vitamin A in µg% 25–60 / Age in years 60–99 / Number of persons 24 | "t" test | Plasma vitamin A in µg% 1–15 / Age in years 16–59 / Number of persons 18 | Plasma vitamin A in µg% 1–15 / Age in years 60–99 / Number of persons 43 | "t" test | Plasma vitamin A in µg% 25–60 / Age in years 16–59 / Number of persons 21 | Plasma vitamin A in µg% 25–60 / Age in years 60–99 / Number of persons 24 | "t" test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | % | | % | % | | % | % | | % | % | | % | % | |
| Localized conjunctival thickening | 21 | 2 | 7.3 | 4 | 0 | 1.0 | 29 | 4 | 3.1 | 4 | 29 | 3.0 | 0 | 4 | 1.0 |
| Blepharo-conjunctivitis | 30 | 16 | 1.8 | 30 | 10 | 1.5 | 31 | 25 | 0.6 | 30 | 31 | 0.1 | 10 | 25 | 1.4 |
| Dryness and/or scaliness of skin | 64 | 42 | 2.3 | 30 | 16 | 1.0 | 81 | 67 | 1.3 | 30 | 81 | 3.9 | 16 | 67 | 4.2 |
| Toad skin | 44 | 9 | 5.0 | 35 | 0 | 3.2 | 50 | 16 | 2.2 | 35 | 50 | 1.1 | 0 | 16 | 2.2 |

$$\text{"t"} = \frac{\text{difference between averages}}{\text{standard error of difference}}.$$

Kruse's conjunctival findings with Bitot's spots was, however, strongly contested by Berliner ('42), who suggested that the conjunctival infiltrations seen by Kruse were merely pingueculae, the changes being limited to the subconjunctival layer and not to the epithelium itself. According to Berliner, other raised spots may also be seen in very old individuals, consisting of yellow concretions of lipoid or hyaline material and white chalky deposits resulting from degeneration of cellular products. In a publication of the same year Kodicek and Yudkin ('42) expressed themselves as being in agreement with Berliner's criticism but suggested that the conjunctival lesion might be a forerunner of the Bitot spots and a sign of vitamin A deficiency. In view of the present uncertainty in the definition of various types of conjunctival spots, and in the absence of conjunctival biopsies, the authors have preferred to include the different observed lesions in one group, which in the table is designated as "localized conjunctival thickening." It appears likely that most of the pericorneal spots observed, although rather similar to the description given above, are not true Bitot's spots, as a large number were located medial to the cornea which is claimed rarely to be the case with the spots observed by Bitot.

Investigations of the correlation between plasma vitamin A values and the presence of conjunctival thickening as observed by slit lamp examination have been reported by Anderson and Milam ('45), who failed to demonstrate any certain relation between the conjunctival changes and the plasma vitamin A level but noted an increased incidence of conjunctival abnormalities with advancing age.

The results of our studies are reported in table 1. It will be seen from the table that the conjunctival thickening occurs with greater frequency in individuals with a low (1–15 μg%) than with a higher (25–60 μg%) plasma vitamin A content. Further analysis of the data indicates, however, that a combination of low plasma vitamin A content and old age appears to be important in the development of the conjunctival thickening.

The occurrence of *blepharoconjunctivitis* was also more frequent in individuals with a low than with a higher plasma vitamin A content, but the difference between the groups was not marked.

*Dryness and/or scaliness of the skin*

This symptom likewise occurred with greater frequency in patients with a low (1–15 μg%) than with a higher (25–60 μg%) plasma vitamin A level. A perusal of the columns in table 1 shows, however, that the effect of age is considerably greater than the influence of the vitamin plasma concentration, the frequency being much greater in the 60–99 year age group than in the 16–59 year old subjects. It should, however, be remembered in this connection that old individuals generally show less inclination for bathing than younger persons, a factor which may have influenced to some extent the above results, tending to aggravate the observed difference between the age groups.

*Toad skin*

The frequent occurrence of follicular hyperkeratosis in vitamin A deficiency was first described by Frazier and Hu ('31). The importance of this sign in the clinical picture of hypovitaminosis A has now been generally accepted, although it is believed that other factors may also be of etiological significance.

As will be seen in table 1, follicular hyperkeratosis was found with greater frequency in patients with a low vitamin A content than in individuals with larger stores of this vitamin, whereas its relation to age is less certain. In agreement with the observations of others, the toad skin was found most commonly on the extensor surfaces of the arms, forearms and thighs; in our observations the buttocks were also frequently affected. In young and middle-aged individuals the lesion consisted of dry, horny, round or oval sharply-defined protuding papules. In old subjects, however, the ap-

pearance of the eruption was somewhat different, the papules being smaller, only slightly elevated, and with a light yellowish or white center. It seems reasonable to assume that this difference was induced by the general atrophy of the skin so frequently present in old age.

### *Dark adaptation*

Although the literature dealing with adaptation time in hypovitaminosis A is very extensive, observations on the correlation between the plasma vitamin A value, determined by a reliable technique, and the adaptation time have been reported only by Pett and Le Page ('40), who studied 26 male subjects between the ages of 20 and 30 years and found a definite relation between the two sets of values. The effect of age on dark adaptation has been studied by Phillips ('39), and by Robertson and Yudkin ('44). In both investigations a highly significant correlation was found, the correlation coefficient in Phillips' studies being + 0.57 to 0.81 and in Robertson and Yudkin's investigations + 0.56. A positive correlation coefficient is indicative of increased adaptation time with age. It was suggested by the latter authors that this impairment of dark adaptation with advancing years was caused by decreased size of the pupils in old individuals.

In the present study the dark adaptation was determined by the Feldman adaptometer.[2] Before being subjected to the test the patients were examined by opthalmoscopy and all individuals with lenticular opacities or other severe ocular lesions were excluded. Further, several patients were omitted because of senile psychosis or dementia. The screening of unfit individuals left only 77 for dark adaptation determinations.

The results, which are reported in figures 1 and 2, show no correlation between the vitamin A content of the plasma and the adaptation time (correlation coefficient —0.01) but a definite, though moderate, correlation between the age and

[2] American Optical Company.

the adaptation time (correlation ratio + 0.41). The latter finding is in agreement with the observations cited above. The reported figures do not permit conclusions concerning the cause of the impaired dark adaptation with age. It seems reasonable to assume that the size of the pupil, as suggested by Robertson and Yudkin, is of importance, but in the opinion of the present authors retinal arteriosclerosis with reduced

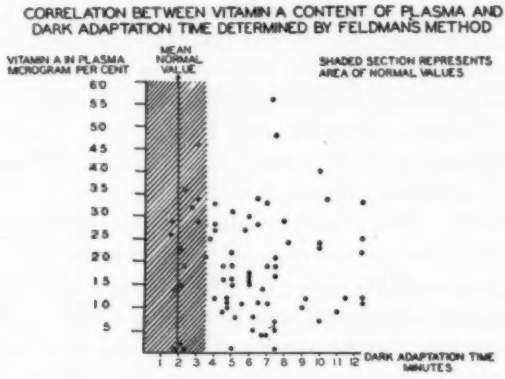


Figure 1

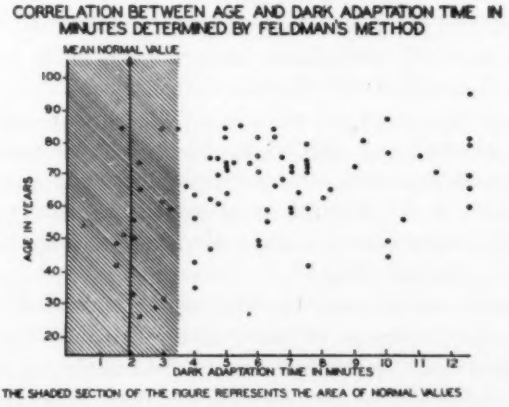


Figure 2

retinal blood supply, and cerebral arteriosclerosis with accompanying prolonged reaction time, cannot be ruled out as contributory factors.

*Keratinized cells in conjunctival epithelium*

According to the experience of Sweet and K'Ang ('35), the examination of conjunctival smears for keratinized epithelial cells constitutes a valuable method for the early diagnosis of hypovitaminosis A. No quantitative data in support of this contention are given, however, in their paper. A significant contribution to the subject was made in 1938 by Youmans, Corlette, Corlette and Frank, who examined conjunctival smears from 25 normal and 9 poorly fed individuals in order to establish criteria for the diagnosis of mild forms of deficiencies. Their results showed no significant difference between the proportion of keratinized cells and the nutritional state of the patient. The average per cent of cornified epithelial cells found was 59 (range 4–97%); the difference between the figures obtained from the two eyes of the same individual was frequently great, averaging 13%, with a range of from 4 to 34%.

In order to correlate, in the present study, the plasma vitamin A level and the percentage frequency of keratinized cells in the conjunctival epithelium, smears were made from both eyes of 83 individuals by rubbing cotton swabs over the conjunctivae of the everted lower lids. The smears were fixed in methyl alcohol and stained by Shorr's trichrome method ('41), a procedure which usually yielded very satisfactory preparations. A differential count was subsequently made of keratinized and non-keratinized cells in the smears from each eye of the same individual.

The results showed an average occurrence of keratinized cells from the right eye of 35% (range 0 to 100%, s.d. 26), and of 38% from the left eye (range 8 to 97%, s.d. 24). The difference between the two eyes of the same individual averaged 9.4% (range 0–37%, s.d. 7.9).

The mean value found for each of the individuals is plotted in figure 3 against the plasma vitamin A level. The results reveal a slight correlation between the plasma vitamin A values and the percentage of keratinized conjunctival cells, the correlation coefficient being —0.25. In view of this rather low degree of correlation and the frequent considerable difference between the values obtained from the two eyes of the same individual, this method of procedure hardly constitutes a useful approach to the clinical examination of patients with regard to plasma vitamin A concentration.

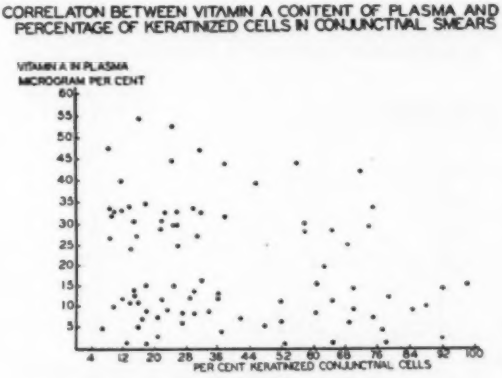


Figure 3

### *Epithelial cells in the urine*

The data in the literature concerning the significance of an abnormal excretion of epithelial cells in the urine and an increased tendency to keratinization of those cells in subjects receiving a diet inadequate in vitamin A are very scarce, and quantitative measurements are entirely lacking. In a 1931 paper Spence comments on the great number of epithelial cells in the urine of children affected with xerophthalmia. In contrast with this, Sweet and K'Ang ('35) in examining fresh urine specimens from 13 children with clinical signs of hypovitaminosis A noted an abnormal number of squamous epithelial cells in only one case.

In the present study measures were undertaken to evaluate quantitatively the excretion of urinary epithelial cells and the frequency percentage of keratinization, and to correlate these findings with the plasma vitamin A concentration.

The sediment count was performed as described by Addis ('25). In consideration of the frequency of urination often encountered in old individuals the collection periods for the urinary samples were, however, often reduced to 2–4 hours, during which time the patients were under constant supervision. In the female patients the samples were collected by

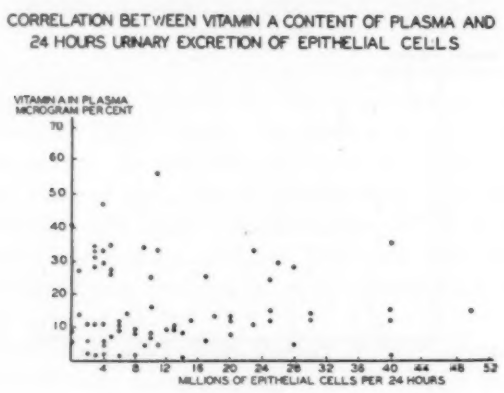


Figure 4

catheterization. The employment of a shorter period for the sample collection was not considered to introduce any error, as investigations by Naeraa ('36) have shown that essentially similar values are found by examination of 24-hour specimens and by calculation of the cellular output on the basis of fractional samples.

Eighty-four individuals were included in the investigation. In 13 cases the presence of urinary infection was noted. The plasma vitamin A levels for the latter individuals were 3, 5, 7, 9, 25, 27, 29, 30, 31, 40, 45, 46, and 48 μg% respectively (average, 25 μg%). As the mean vitamin A plasma concen-

tration for the 71 subjects without evidence of urinary infection was 15 μg%, these findings do not support the view that a low vitamin A content of the plasma is a factor of significance in the development of urinary infection in humans. The epithelial counts observed in the 71 cases are reported in figure 4. The data disclose no correlation between the plasma vitamin A value and the number of excreted epithelial cells, the correlation coefficient being —0.02.

Smears of the urinary sediment were fixed and stained by the same technique employed in the investigation of the con-

CORRELATION BETWEEN VITAMIN A CONTENT OF PLASMA AND PERCENTAGE OF KERATINIZED CELLS IN URINARY SEDIMENT

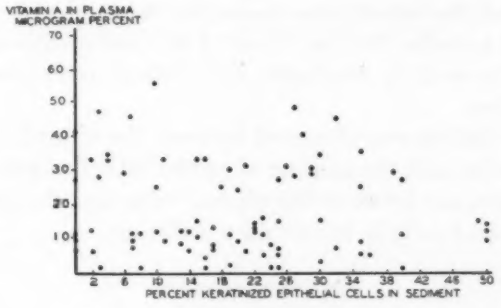


Figure 5

junctival samples. The keratinized cells were usually clearly distinguishable and could easily be subjected to differential counting. The results of the counts in the 71 samples which showed no evidence of infection are given in figure 5. The data show no certain correlation between the vitamin A value and the percentage of keratinized cells, the correlation coefficient being —0.20.

## SUMMARY

A clinical study of 106 individuals with regard to signs of vitamin A deficiency revealed a considerably higher frequency of occurrence of hyperkeratosis of the skin follicles

and of localized conjunctival thickening in the subjects with a low vitamin A plasma level (1–15 μg%) than in those having a higher plasma vitamin concentration (25–60 μg%). The occurrence of dryness of the skin and of blepharoconjunctivitis was also more frequent in the subjects with hypovitaminemia A, but the difference between the groups was not marked.

No correlation was found between dark adaptation time and vitamin A plasma values but a significant, though moderate, correlation was observed between length of adaptation time and age.

The percentage of keratinized cells in conjunctival smears was slightly higher in indviduals with low vitamin A plasma values, but the percentage variation between different subjects and between the two eyes of the same subject was so great as to tend to invalidate the clinical usefulness of this examination.

No correlation was observed between the vitamin A plasma concentration and the number of epithelial cells excreted daily in the urine, nor between the plasma value and the percentage of keratinized cells in the urinary sediment.

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# SELF SELECTION OF DIET

## IX. THE APPETITE FOR THIAMINE [1]

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The concept of a learned or "educated" appetite was first advanced by Harris, Clay, Hargreaves and Ward ('33). Early studies were made by these workers on "vitamin B," while their later work was concerned with partially purified "vitamin $B_1$." It appears probable that their conclusions are applicable chiefly to thiamine. These investigators attempted to determine the limitations of a learned appetite, and discussed the possibility that such appetites may be of considerable importance in practical feeding. With the advantage of much more complete knowledge of the rat's nutritional requirements, this laboratory has attempted to investigate the nature of learned appetites for thiamine and other B vitamins and to evaluate their significance. This has resulted in the demonstration of such appetites (Scott and Quint, '46b), and in exploration of the role of learning in their inception (Scott and Verney, '47). The present paper describes certain experiments on the appetite of rats for thiamine-containing foods which attempt further to describe and explain the nature of a learned appetite.

### EXPERIMENTAL

In the first series of 10 experiments the effect was studied of thiamine concentration, both in the previous diet and in

[1] This research was aided by grants from the Nutrition Foundation, Inc., and from the Buhl Foundation.

the thiamine-containing choices, on the appetite for thiamine-containing foods. The experimental method was that previously described in detail by Scott and Quint ('46a). In each experiment, 5 male and 5 female weanling rats in separate cages were offered during a three-week control period, a standard purified diet in each of two cups, while another similar group was given in the same way a thiamine-containing diet.[2] These 10 experiments differed among themselves only in the thiamine content of the latter diet. Other vitamins were given separately as pills.[3] Body weight and food consumed from each cup were determined daily and the cups then interchanged in a predetermined random manner. During a three-week experimental period both groups were given their choice, in separate cups, of the standard diet and the same thiamine-containing diet as was used in the control period. Body weight and food consumption from each cup were measured and the cups interchanged daily in the same manner as before.

To determine the effect of additional thiamine-free choices on the appetite for thiamine-containing food, an experiment similar to those above was devised in which, during the experimental period, three cups were filled with standard diet and only one with thiamine-containing diet (two μg per gram).

For reasons discussed below it was decided to study the eating behavior of rats when (1) a high level of thiamine in the diet was first associated with a flavor, or lack of it, and (2) when the flavor was changed with respect to the vitamin. The technique was exactly that previously described in detail (Scott and Verney, '47) except that the thiamine content of the diet was much higher. At the end of the control period one

[2] The standard diet consisted of 24% casein (Labco "vitamin-free"), 10% hydrogenated vegetable oil ("Primex"), and 4% salt mixture (Jones and Foster, '42). Thiamine-containing diets contained in addition 0.1 to 1000 μg/gm of thiamine hydrochloride.

[3] One pill was given each rat daily. It contained approximately 100 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 10 mg choline chloride, 1 mg α-tocopherol, and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola, all in a dextrin-powdered sugar base.

group of 10 thiamine-deficient animals and another of 10 control animals (previously fed 100 μg per gram thiamine in their food) were subdivided and allowed their choice of (1) unflavored standard diet and flavored (10 p.p.m. oil of anise) thiamine-containing (100 μg per gram) diet, or (2) flavored standard diet and unflavored thiamine-containing diet. After 5 days the flavor was reversed with respect to the vitamin, so that animals previously offered choices (1) above now chose from (2), and those previously offered (2) received (1). After this second period of 5 days the original choices were offered for a third period of the same length. During a fourth period of 6 days the choices were the same as in the second period. The amounts of the vitamin-containing and vitamin-free diets consumed in each period were measured.

The final experiment was concerned with determining how deficient in thiamine an animal must be in order to have a definite appetite for thiamine-containing food. Ten male and 10 female weanling rats were divided into two groups so that each animal had a litter-mate pair of the same sex and weight in the other group. One group throughout a 6-week period was given its choice of a thiamine-containing diet (0.5 μg per gram) and the standard diet. The other group was given the same thiamine-containing diet in each of two cups. Body weight and food consumed from each cup were determined and the cups interchanged daily in a predetermined random manner.

## RESULTS

The effects of thiamine concentration on appetite for thiamine-containing food are shown in table 1. The animals fed thiamine-containing diets (control groups) showed no evidence of deficiency in the control period if the thiamine concentration was 1 μg per gram or more, and no appetite for thiamine-containing food in the experimental period if the concentration was 2 μg per gram or more. At the highest level (0.1%) of thiamine, the diet containing the vitamin was avoided. The deficient groups all preferred the thiamine-

TABLE 1

*Appetite for thiamine-containing food* [1]

| EXPT. NO. | THIAMINE [2] CONCENTRATION IN FOOD | CONTROL PERIOD (3 WEEKS) | | | EXPERIMENTAL PERIOD (3 WEEKS) | | | |
|---|---|---|---|---|---|---|---|---|
| | | Weight change | Food eaten | Thiamine [2] intake: average | Weight [3] change | Food eaten | Thiamine [2] intake: average | Appetite [4] for thiamine |
| | *μg/gm* | *gm* | *gm* | *μg/day* | *gm* | *gm* | *μg/day* | % |
| | | | | Control animals | | | | |
| 1 | 0.1 | 22.4 ± 2.3 | 88.5 ± 4.3 | 0.4 | (10) | (14.3 days) | 0.1 | 26.0 ± 9.2 |
| 2 | 0.2 | 28.5 ± 3.5 | 113.0 ± 6.3 | 1.1 | — 29.4 ± 4.7(5) | 47.7 ± 5.0 | 0.3 | 31.5 ± 8.2 |
| 3 | 0.5 | 51.4 ± 3.2 | 129.2 ± 7.9 | 3.1 | — 22.3 ± 5.4 | 84.3 ± 9.4 | 1.6 | 30.4 ± 3.6 |
| 4 | 1 | 66.9 ± 4.8 | 154.9 ± 11.3 | 7.4 | 18.3 ± 6.6 | 151.9 ± 12.5 | 6.0 | 29.7 ± 8.8 |
| 5 | 2 | 72.9 ± 4.0 | 153.9 ± 8.2 | 14.7 | 60.1 ± 4.4 | 237.3 ± 8.7 | 14.3 | 8.3 ± 4.0 |
| 6 | 5 | 63.9 ± 4.6 | 155.2 ± 9.8 | 37.0 | 39.3 ± 6.3 | 199.1 ± 14.5 | 27.3 | 8.8 ± 6.9 |
| 7 | 10 | 65.5 ± 2.8 | 137.5 ± 7.3 | 65.5 | 35.8 ± 3.4 | 182.4 ± 10.7 | 42.1 | — 7.3 ± 7.9 |
| 8 | 20 | 63.0 ± 4.6 | 141.3 ± 7.0 | 134.5 | 58.9 ± 5.3 | 216.8 ± 10.9 | 87.6 | — 6.3 ± 7.2 |
| 9 | 100 | 61.3 ± 2.4 | 147.3 ± 6.5 | 701.4 | 64.6 ± 5.5 | 246.5 ± 9.5 | 753.7 | 10.7 ± 8.4 |
| 10 | 1000 | 70.6 ± 2.5 | 157.6 ± 4.3 | 7504.8 | 52.0 ± 4.5 | 223.4 ± 7.4 | 2724.0 | — 22.3 ± 4.3 |
| | | | | Experimental animals | | | | |
| 1 | 0 | 17.5 ± 3.3 | 82.2 ± 3.8 | 0 | (9) | (12.0 days) | 0.1 | 16.7 ± 9.7 |
| 2 | 0 | 11.3 ± 2.6 | 85.0 ± 6.3 | 0 | (10) | (11.6 days) | 0.2 | 7.1 ± 8.0 |
| 3 | 0 | 29.9 ± 1.7 | 99.6 ± 5.2 | 0 | — 15.1 ± 3.2 | 72.1 ± 4.9 | 1.3 | 20.9 ± 8.7 |
| 4 | 0 | 18.7 ± 2.0 | 93.1 ± 5.7 | 0 | 18.2 ± 6.9(4) | 90.4 ± 9.0 | 3.1 | 35.6 ± 5.8 |
| 5 | 0 | 22.2 ± 3.0 | 91.0 ± 5.6 | 0 | 74.1 ± 5.4(3) | 188.3 ± 14.2 | 11.1 | 19.7 ± 10.1 |
| 6 | 0 | 15.6 ± 2.2 | 94.0 ± 4.6 | 0 | 77.3 ± 4.5 | 189.2 ± 7.6 | 36.0 | 29.8 ± 5.2 |
| 7 | 0 | 20.4 ± 1.6 | 84.8 ± 3.2 | 0 | 56.9 ± 2.9 | 177.0 ± 8.4 | 65.8 | 23.8 ± 11.8 |
| 8 | 0 | 14.4 ± 3.4 | 86.8 ± 3.5 | 0 | 64.2 ± 4.8 | 159.9 ± 10.7 | 98.4 | 23.0 ± 5.7 |
| 9 | 0 | 20.4 ± 2.4 | 95.2 ± 5.0 | 0 | 81.1 ± 3.5 | 211.8 ± 8.0 | 776.7 | 29.8 ± 5.8 |
| 10 | 0 | 17.5 ± 2.2 | 87.5 ± 3.5 | 0 | 72.8 ± 3.2 | 192.4 ± 6.1 | 7678.8 | 41.1 ± 7.5 |

[1] All data are in terms of the mean and the standard error of the mean.

[2] Actually, thiamine hydrochloride.

[3] Figures in parentheses represent number of animals that died in the experimental period. These animals were excluded from the weight change and food intake data. Where the majority of animals died the figure given is the average length of life of the group in the experimental period.

[4] Average difference (in per cent of total food intake) between the intake of vitamin-containing food in the experimental period and the food intake from the corresponding cup in the control period. A significant positive value indicates a preference.

TABLE 2

*Preference for thiamine-containing food as one choice out of 4*

| | CONTROL PERIOD | | EXPERIMENTAL PERIOD | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Preferences [1] for | | | |
| | Weight change | Food eaten | Weight change | Food eaten | Thiamine-containing choice | Other choices | | |
| | *gm* | *gm* | *gm* | *gm* | % | % | | |
| Control animals | 78.0 ± 3.4 | 158.4 ± 5.6 | 66.1 ± 5.3 | 254.6 ± 10.4 | 43.4 ± 3.8 | — 17.8 ± 7.7 | — 15.8 ± 2.2 | — 10.7 ± 1.9 |
| Deficient animals | 21.6 ± 2.5 | 93.2 ± 4.4 | 68.1 ± 6.0 | 171.1 ± 10.9 | 54.8 ± 7.5 | — 23.0 ± 2.7 | — 20.5 ± 3.4 | — 12.4 ± 2.9 |

[1] Average difference (in per cent of total food) between the food consumed from a given cup in the control period and that consumed from the same cup in the experimental period. A positive value indicates a preference.

containing diet although, at very low thiamine concentrations, the condition of the rats was so poor that it may have made the results uncertain.

The results of the experiment in which the thiamine-containing food was one choice among three are presented in table 2. Both the control and deficient groups uniformly selected the thiamine-containing choice.

In the previously reported flavor study of thiamine (Scott and Verney, '47) it was found that the normal animals which

TABLE 3

*Flavor change and preference for thiamine*

| THIAMINE LEVEL IN DIET | PART OF EXPERIMENTAL PERIOD | | | |
|---|---|---|---|---|
| | First | Second | Third | Fourth |
| *p.p.m.* | | | | |
| | Difference in body weight change [2] (gm per day) | | | |
| 5 [1] | −0.30 ± 0.56 | −1.16 ± 0.73 | 0.46 ± 0.54 | 1.25 ± 0.46 |
| 100 | 0.86 ± 0.54 | 0.16 ± 0.42 | −0.02 ± 0.56 | −0.18 ± 0.45 |
| | Difference in preference for thiamine [3] (per cent) | | | |
| 5 [1] | 57.2 ± 8.2 | −11.0 ± 14.7 | 30.4 ± 12.0 | −1.1 ± 13.6 |
| 100 | 47.0 ± 10.7 | −15.4 ± 20.4 | 8.8 ± 12.5 | −11.8 ± 12.3 |

[1] Data from Scott and Verney ('47).

[2] Average weight change of deficient animals minus that of control group.

[3] Average per cent thiamine-containing food selected by deficient animals minus per cent selected by control animals. A significant positive value indicates a greater preference by the deficient animals.

had received thiamine in the control period showed little or no preference in the experimental period. Accordingly it appeared proper to use them as a control group to which the deficient animals could be compared. This was done, as is shown in table 3, where the data from experiments using both a high level (100 μg per gram) and low level (5 μg per gram) of thiamine as a choice are summarized. In both experiments there was a pronounced preference by deficient animals for the thiamine-containing diet in the first part of the experimental period, and a slight avoidance of it in the second part

after the flavor was switched with respect to the vitamin. The results of the two experiments differed in the third part, with the deficient animals on the low-thiamine choice showing a distinct preference while those at the higher level showed only a slight preference. In the fourth part both deficient groups avoided the thiamine-containing diet to a slight extent. The differences in weight gains show a more rapid re-

TABLE 4

*Comparison of animals with and without choice of thiamine-containing food*

| WEEK | DIFFERENCE BETWEEN PAIRS | | | |
|---|---|---|---|---|
| | Wt. change [1] | Food eaten [1] | Thiamine intake [1] | Preference [2] |
| | *gm/wk.* | *gm/wk.* | *μg/day* | % |
| First | 2.7 ± 1.2 | 1.6 ± 1.5 | 1.3 ± 0.3 | 9.9 ± 11.4 |
| Second | 6.9 ± 2.4 | 10.0 ± 2.7 | 2.3 ± 0.4 | — 3.6 ± 9.7 |
| Third | 11.8 ± 4.3 | 18.3 ± 6.8 | 1.9 ± 0.6 | 28.4 ± 11.5 |
| Fourth | 10.9 ± 4.7 | 25.3 ± 8.3 | 2.1 ± 0.7 | 35.5 ± 10.3 |
| Fifth | 8.8 ± 5.2 | 21.7 ± 10.6 | 1.7 ± 0.8 | 25.7 ± 10.5 |
| Sixth | 6.0 ± 3.7 | 15.6 ± 9.4 | 1.3 ± 0.7 | 29.8 ± 8.1 |

[1] Average of the figures for the animals fed only a thiamine-containing diet minus the figures of the corresponding pair mates given a choice. A positive value indicates greater weight gain, food consumption, or thiamine intake for the animals without choice.

[2] Average per cent of total food that was thiamine-containing selected by animals on choice minus the per cent of total food eaten from a corresponding cup by respective pair mates not given a choice. A significant positive value indicates a preference for thiamine-containing food by the animals given a choice.

covery of the animals selecting a high concentration of thiamine.

In the final experiment weekly differences between pairs of animals, one of which had a choice of thiamine-containing and thiamine-free diets and the other only a thiamine-containing diet, were compared (see table 4). It will be noted that the animals given a choice grew significantly less as early as the first week and that they ate less food in the second week, but that not until the third week was a preference shown by them for the thiamine-containing food.

## DISCUSSION

The thiamine appetite has been called a learned appetite, but it should be emphasized that the reason for this type of food preference is at best obscure. A learned appetite can be defined only as an appetite which, like the appetite for thiamine, is (1) shown by deficient but not by normal animals and is (2) capable of being associated experimentally with characteristics of the diet not affected by the presence or absence of the nutritional essential. As the thiamine appetite may be considered the prototype of learned appetites, many of the conclusions regarding it can be expected to apply to other learned appetites.

The experiments on concentration indicate that an appetite for thiamine-containing foods is present at levels of thiamine so low that its benefit for growth is scarcely perceptible, and also at levels where it exceeds the growth requirement by a factor of at least 100. In order to establish that a learned appetite may exist it has been assumed that normal animals must show distinctly less preference for a diet containing an essential than deficient animals do. It appears from our concentration experiments that the level of the dietary essential can be too low to demonstrate a difference between the preferences of normal and deficient animals, but that it cannot be too high. This would be expected to be generally true, except when the essential is characterized by a flavor which affects the animals' choice.

It was indicated in our earlier experiments on the relation between flavor and thiamine (Scott and Verney, '47) that prefence for thiamine was due both to a continuing perception of a feeling of well-being on eating the thiamine-containing food and to an established habit of eating that food. This conclusion was reached because in the second part of the experimental period, after the flavor was first changed with respect to the vitamin, the prefence of the animals for the flavor that had been originally associated with the vitamin-containing diet was not as strong as it was when combined with thiamine in the first and third periods. If, however, the

thiamine level were high, it might be anticipated that the feeling of well-being experienced by the animal on eating the diet would not continue long, since recovery from deficiency would be more rapid. This appeared to be the fact, since, in the flavor experiment using a high level of thiamine, only in the first part of the experimental period was there a marked preference for thiamine-containing food indicative of a perception of a feeling of well-being. Thus the results were consistent with the existence of dual causes of continued preference for vitamin-containing diets, wherein a feeling of well-being on eating the diet was perceived by deficient animals and a habit of eating food characterized by the flavor originally associated with thiamine was established by them. The perception of a feeling of well-being persisted longer at the low thiamine level than at the high level, while the habit was established in either case.

Thiamine-deficient rats had no difficulty in finding the thiamine-containing food when it was offered as one of 4 choices and, in fact, offering more choices made the difference between random eating and the case where choice was shown more obvious. It should be noted that the control animals chose thiamine when 4 choices were offered, whereas they had not when two choices were given. This appeared to have its cause in the obvious circumstance that at the same thiamine content of the diet the actual thiamine intake is lower in random eating, when more thiamine-free choices are added. As a result, the control animals with 4 choices became deficient early in the experimental period and then chose the food containing thiamine, while those animals with two choices received a higher total thiamine intake by random eating and did not become deficient.

The final experiment was designed to determine how deficient animals must be to have distinct appetities for thiamine-containing foods. From table 4 it is apparent that both weight change and food consumption are more sensitive criteria of deficiency in point of time than is preference for thiamine. By the time a distinct preference was made by the animals

given a choice, they were in so much poorer condition than the controls that they could not catch up with their pair mates. The effect of their choice under these circumstances did not approach that which would result from the prescription of a good diet in promoting adequate nutrition.

In an evaluation of the possible importance in practical nutrition of the appetite for thiamine the following points should be considered:

(1) The appetite for thiamine is shown only by animals in a state of thiamine deficiency. There is no evidence of an "instinctive" appetite for thiamine. Thiamine appetite can lead to remission from thiamine deficiency but does not prevent its onset.

(2) The appetite for thiamine is not universal in all rats. Of 80 animals in these experiments who were previously fed thiamine-free diets and then offered a choice of a thiamine-free diet and one containing 1 p.p.m. or more of thiamine, two animals ate less than 10% of their total food from the thiamine-containing choice and 7 ate less than 50% of their food from that choice.

(3) A simple uncomplicated choice of the type obtained in these experimental studies will not ordinarily occur in actual feeding. Natural foods different in thiamine content will ordinarily be of different origins and will vary widely in flavor and in physical and chemical properties. Several other animals are much more selective in choice of food than are rats, and are markedly influenced by flavor. Food choice in man in particular is influenced by a large number of social, cultural and economic factors which, with the exception of education based on scientific nutrition, appear to bear little if any relation to nutritional needs. Thus an appetite for thiamine under most actual feeding conditions can scarcely be anticipated on the basis of our studies.

It appears, then, that a learned appetite for thiamine under certain conditions can lead to better nutrition than would wholly random eating, but that it cannot result in better nutrition than when the diet is prescribed on the basis of known

nutritional needs. In fact, under critical conditions such as are found in the final experiment above, self selection of diet for thiamine would ordinarily be inferior to obligatory feeding of thiamine-containing foods in promoting nutritional well-being.

## SUMMARY

Rats suffering from thiamine deficiency show a preference for diets containing as little as 0.1 and as much as 1000 p.p.m. of thiamine. The appetite for thiamine-containing foods was not universal in thiamine-deficient rats and a small minority always made a poor choice. Rats fed a diet with a critically low concentration of thiamine were in better condition than rats offered a choice between this diet and a thiamine-free diet. Appetite for thiamine-containing foods was not established until the animals were distinctly deficient as judged by weight gain and food intake. It was concluded that while the learned appetite for thiamine can lead to better nutrition than would wholly random eating, it can at best only approach, and under critical conditions fails to approach, the benefit obtained by obligatory eating of a diet prescribed on the basis of known requirements.

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# PHLORHIZIN DIABETES IN VITAMIN DEFICIENCIES [1]

## CONVERSION OF PROTEIN TO GLUCOSE, AND SERUM PHOSPHATASE, DURING DEFICIENCIES OF THIAMINE, RIBOFLAVIN, AND PYRIDOXINE

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### INTRODUCTION

Conversion of amino acids to glucose is a rather intricate phase of protein metabolism. Deamination, which is the first step, may be effected by way of transaminase and L-glutamic dehydrogenase. The latter enzyme requires coenzyme I, containing nicotinic acid. Transaminase activity in tissues is diminished during pyridoxine deficiency (Schlenk and Snell, '45), and the relationship appears to be a direct one (Ames, Sarma and Elvehjem, '47). McHenry and Gavin ('41), discussing the formation of fat from protein, suggested that pyridoxine may be required for preliminary conversion of protein to carbohydrate. Riboflavin deficiency might be expected to interfere with oxidation of glucose formed from protein, but no evidence can be cited in this connection.

These considerations, together with an interest in the effects of vitamin deficiencies in experimental pancreatic diabetes (Gaebler and Ciszewski, '45; Gaebler and Mathies,

[1] Data in this paper are taken from Part I of a thesis submitted by one of the authors (P.D.B.) to the Graduate Council of Wayne University in partial fulfillment of requirements for the degree of Doctor of Philosophy in Chemistry.

'46), led us to study the effects of thiamine, riboflavin, and pyridoxine deficiencies on the D:N ratio in phlorhizin diabetes. Nicotinic acid deficiency could not be included, since we used a diet containing over 72% of vitamin-free casein, which would provide for maximum synthesis of nicotinic acid from tryptophane. Since formation of glucose from protein takes place in the liver, several liver function tests were included in our studies. As a result, we obtained unexpected data on changes in the alkaline phosphatase of serum during phlorhizin diabetes.

## EXPERIMENTAL

Adult female dogs were given a diet having the following percentage composition: Vitamin-free casein,[2] 72.2; corn oil, 19.8; Phillips-Hart ('35) salt mixture, 4; calcium phosphate, 4. Each animal received daily three drops of haliver oil, 500 mg of choline chloride, and the following amounts, in μg per kilo, of other vitamins: thiamine HCl, 50; riboflavin, 100; nicotinic acid, 500; pyridoxine HCl, 40; calcium pantothenate, 200. After a preliminary period of caloric adjustment, one vitamin was omitted. When characteristic signs of deficiency appeared, 1 gm of phlorhizin, finely suspended in olive oil, was injected morning and evening. In 5 experiments the deficiency was terminated after the third or fourth day of phlorhizin by administering large amounts of the missing vitamin. Phlorhizination and determination of D:N ratios were continued during the dramatic recovery which followed.

Urines were collected under toluene in 24-hour periods terminated by catheter. Nitrogen was determined by Kjeldahl digestion of samples containing about 5 mg, followed by distillation into 0.02 N acid and titration with 0.02 N alkali. Glucose was determined according to the method of Shaffer and Somogyi ('33) after diluting the urines 1:100. True sugar in zinc hydroxide filtrates of blood (Somogyi, '30) was determined with the same reagent. Hemoglobin values, of special interest in pyridoxine and riboflavin deficiencies, were deter-

[2] Labco.

mined with a Beckman spectrophotometer calibrated on the basis of oxygen capacity. Bromsulphalein in serum was determined in the manner described by one of the authors (Gaebler, '45), and alkaline phosphatase by the method of Bodansky ('33). The method of Van Allen ('25) was used for cell volume and that of Whitehorn ('21) for serum chlorides.

## RESULTS

Dog 38 took the pyridoxine-free diet greedily for 18 days, but refused increasing amounts of it after 23 days. In spite of this, no anemia developed. On the 34th day the animal became completely incoordinated during play, and later had violent convulsions. The true sugar concentration in its blood just before this episode was 71 mg%. Normal appetite returned within 24 hours after 10.4 mg of pyridoxine were injected, and all untoward symptoms disappeared. Depletion was started a second time after the dog had, for 11 days, received the complete vitamin supplement already described. During 18 days of depletion the weight declined from 14.72 to 14.38 kg, increasing amounts of food were refused, and nitrogen balances became steadily more negative. All food was refused during phlorhizination, which was begun at the end of this period. As shown in table 1, the D:N ratios were quite low on the third and 4th days of phlorhizin administration but rose to 3.42 and 3.65, or an average of 3.54, when pyridoxine was given. Since the dog was consuming its own tissues, it may be pointed out that this value is in good agreement with the D:N of 3.49 reported by Janney and Csonka ('15) for dogs maintained on a diet consisting of dog muscle proteins.

Although dog 40 took the pyridoxine-free diet constantly and in adequate amount, its weight decreased steadily. Hemoglobin values fell uniformly, from 15.2 gm% on the 15th to 11.0 on the 30th day of depletion. On and after the 22nd day, 1 gm of desoxypyridoxine [3] was fed daily. Phlorhizination was begun on the 28th day. The D:N ratios are shown in

[3] We are indebted to Merck and Company for a liberal supply of this antivitamin, which is 2,4-dimethyl-3-hydroxy-5-hydroxymethyl pyridine.

table 1. During the first three phlorhizin days the animal refused about half of its food. When the antivitamin was stopped and pyridoxine was given, the dog resumed eating all of its food and the ratios rose. That the ratios on the second and third days of phlorhizin, during deficiency, were somewhat low is also indicated by reference to the control experiment shown in the last column of table 1. When this experiment was begun, dog 40 had, for 25 days, received a normal amount of pyridoxine.

TABLE 1

*D:N ratios in phlorhizinized dogs during and after pyridoxine deficiency*

| DAY OF PHLORHIZIN INJECTION | DOG 38 DURING AND AFTER PYRIDOXINE DEFICIENCY | DOG 40 DURING AND AFTER PYRIDOXINE DEFICIENCY | DOG 40 LONG AFTER PYRIDOXINE DEFICIENCY |
|---|---|---|---|
| 1 | 3.49 | 2.67 | 2.61 |
| 2 | 3.04 | 2.90 | 3.44 |
| 3 | 2.70 | 3.01 | 3.28 |
| 4 | 2.79 | 3.21 [2] | 3.18 |
| 5 | 3.42 [1] | 3.26 | 2.96 |
| 6 | 3.65 | 3.24 | 3.18 |

[1] Forty milligrams of pyridoxine were injected subcutaneously on the 5th and 20 mg on the 6th phlorhizin day.

[2] Feeding of desoxypyridoxine was discontinued. Twenty milligrams of pyridoxine were injected and 60 mg given by mouth. On and after the 5th phlorhizin day the vitamin supplement was complete.

The significant data of a riboflavin deficiency experiment on dog 40 are condensed in table 2. During the first 37 days, while food consumption was complete, the weight actually rose; thereafter it declined irregularly. Evidence of itching, and seborrhea, appeared as early as the 20th day; anorexia, on the 38th day. A 5-day phlorhizin period was begun on the 39th day and a second 6-day period on the 57th day of deficiency. At the latter time hemoglobin had fallen to 10.9 gm%. After the second phlorhizin period all deficiency symptoms became severe. Food was refused completely after the 68th day, the hemoglobin was 9.1 gm%, itching and seborrhea were

TABLE 2

*D:N ratios, hemoglobin, serum phosphatase, and bromsulphalein retention in phlorhizin diabetes during riboflavin deficiency*

| TIME AFTER WITHDRAWAL OF RIBOFLAVIN | WEIGHT | FOOD CONSUMPTION | URINE NITROGEN PER DAY | URINE SUGAR PER DAY | D:N RATIO | HEMOGLOBIN | CELL VOLUME | SERUM PHOSPHATASE (BODANSKY, '33) | BROMSULPHALEIN RETENTION |
|---|---|---|---|---|---|---|---|---|---|
| *days* | *kg* | *% of normal* | *gm* | *gm* | | *gm%* | *%* | *units* | *%* |
| 8 | 9.98 | 100 | | | | 14.90 | 42.5 | | |
| 11 | 10.02 | 100 | | | | 14.70 | 41.3 | | |
| 14 | 9.98 | 100 | | | | 14.95 | 43.5 | | |
| 23 | 10.13 | 100 | | | | 14.50 | 42.0 | 5.6 | |
| 29 | 10.24 | 100 | | | | 14.95 | 43.1 | 5.5 | |
| 37 | 10.46 | 100 | | | | 13.80 | 40.0 | | |
| 38 | 10.02 | 50 | | | | | | | |
| 39 [1] | 10.13 | 100 | 22.13 | 45.51 | 2.06 | | | | |
| 40 | 9.79 | 50 | 21.60 | 53.80 | 2.49 | | | | |
| 41 | 9.68 | 50 | 17.50 | 57.23 | 3.27 | | | 12.5 | |
| 42 | 9.57 | 100 | 22.99 | 66.88 | 2.91 | | | | 5.2 |
| 43 | 9.68 | 100 | 22.00 | 64.80 | 2.94 | | | 11.1 | |
| 49 | 9.90 | 100 | | | | 12.4 | 37.6 | 8.7 | |
| 56 | 9.83 | 83 | | | | 10.9 | 32.5 | 7.7 | 3.2 |
| 57 [2] | 9.57 | 50 | 13.05 | 43.00 | 3.30 | | | | |
| 58 | 9.57 | 50 | 14.50 | 46.40 | 3.20 | | | | |
| 59 | 9.23 | 50 | 16.60 | 52.00 | 3.13 | | | | |
| 60 | 9.12 | 50 | 16.78 | 55.60 | 3.30 | | | | |
| 61 | 9.23 | 100 | 21.35 | 63.60 | 2.98 | | | | |
| 62 | 9.12 | 100 | 22.55 | 65.70 | 2.91 | | | | |
| 63 | 9.12 | 100 | | | | | | 5.4 | |
| 66 | 9.12 | 91 | | | | 9.10 | 29.0 | 5.6 | |
| 68 | | 0 | | | | | | | |
| 75 [3] | 9.19 | 100 | | | | 9.10 | 29.0 | | |
| 87 | 9.76 | 100 | | | | 11.75 | 35.8 | | |
| 93 | 9.76 | 100 | | | | 12.20 | 36.0 | | |

[1] One gram of phlorhizin injected subcutaneously at 9 A.M. and again at 5 P.M., 39th to 43rd day.

[2] Phlorhizination repeated in the above manner, 57th to 62nd day.

[3] One hundred milligrams of riboflavin given by mouth daily on the 75th, 76th, and 77th days; complete supplement given thereafter.

marked, loss of hair around the eyes produced the "spectacle eye" appearance, and there was evidence of photophobia. All changes reversed after feeding of riboflavin was resumed on the 75th day. The D:N for the third, 4th, and 5th phlorhizin days averaged 3.04 in the first experiment and 3.14 in the second. The ratio reported by Janney ('15) for dogs on casein diets is 3.08. Bromsulphalein retention of the 5 mg per kilo dose at 30 minutes showed no evidence of liver damage. The upper limit of 15% retention in normal dogs reported by Drill and Ivy ('44) corresponds to 6% in our terms, since they used the 5 mg per kilo dose of bromsulphalein but preferred the standards corresponding to 2 mg per kilo. The rise in alkaline phosphatase shown in table 2 is clearly associated with phlorhizination, not with riboflavin deficiency, and will be discussed later.

Results of a riboflavin deficiency experiment in another animal, dog 43, are shown in table 3. Since loss of weight and nitrogen began almost immediately after riboflavin was withdrawn, the food intake was increased 25% after the first 12 days. Although the weight was stable for the next 18 days, nitrogen loss continued after a single three-day period. Anorexia and vomiting started on the 42nd day, but the animal resumed eating and phlorhizin was started on the 48th day. In the previous experiment we compared D:N ratios of two phlorhizin periods representing moderate and severe riboflavin deficiency; in the present experiment, the deficiency was terminated during the phlorhizin period. The ratios for the third and 4th phlorhizin days, during deficiency, averaged 3.26; those for the 5th and 6th, after riboflavin therapy was begun, averaged 3.19. The rise in serum phosphatase confirms the finding in the previous experiment.

Observations made during thiamine deficiency are recorded in table 4. Dog 43 lost weight steadily, vomited on the 23rd and 24th days of depletion, and was unable to retain either food or water on the 28th day. Dramatic improvement occurred on the 29th day when thiamine was injected. Food was consumed and retained, and the animal was active during

TABLE 3

*Nitrogen equilibrium, glucogenesis, and other functions during riboflavin deficiency*

| TIME AFTER WITHDRAWAL OF RIBOFLAVIN | WEIGHT | FOOD CONSUMPTION | NITROGEN BALANCE PER DAY | URINE NITROGEN PER DAY | URINE SUGAR PER DAY | D: N RATIO | HEMOGLOBIN | CELL VOLUME | SERUM PHOSPHATASE (BODANSKY, '33) |
|---|---|---|---|---|---|---|---|---|---|
| *days* | *kg* | *% of normal* | *gm* | *gm* | *gm* | | *gm%* | *%* | *units* |
| 1–3 | 13.01 | 100 | —0.18 | | | | | | |
| 4–6 | 12.89 | 100 | —1.02 | | | | | | |
| 7–9 | 12.75 | 100 | —0.79 | | | | | | |
| 10–12 | 12.71 | 100 | —0.85 | | | | | | |
| 13–15 | 12.55 | 125 [1] | 0.70 | | | | | | |
| 16–18 | 12.71 | 125 | —0.90 | | | | | | |
| 19–21 | 12.75 | 125 | —0.13 | | | | | | |
| 22–24 | 12.71 | 125 | —1.24 | | | | | | |
| 26 | 12.67 | 125 | | | | | 15.7 | 43.0 | |
| 30 | 12.59 | 125 | | | | | 15.3 | 44.0 | |
| 46 | 12.03 | 125 | | | | | 16.8 | 47.9 | 9.0 |
| 47 | 12.14 | 125 | | | | | 13.4 | 39.0 | 6.5 |
| 48 [2] | 11.91 | 63 | | 13.70 | 34.60 | 2.52 | | | |
| 49 | 11.80 | 0 | | 9.05 | 30.25 | 3.34 | | | |
| 50 | 11.57 | 0 | | 8.38 | 27.95 | 3.34 | | | |
| 51 | 11.12 | 0 | | 8.55 | 27.15 | 3.18 | 14.1 | 40.5 | 11.0 |
| 52 [3] | 10.88 | 63 | | 10.12 | 32.48 | 3.21 | | | |
| 53 | 10.88 | 125 | | 14.60 | 46.24 | 3.17 | | | |
| 54 | 11.12 | 125 | | 15.84 | 47.92 | 3.02 | | | |
| 55 | 11.08 | 125 | | | | | 13.3 | 40.0 | 7.4 |
| 65 | 11.00 | 125 | | | | | 13.0 | 37.0 | 4.3 |

[1] Food intake increased in an attempt to reverse negative nitrogen balances.

[2] One gram of phlorhizin injected twice daily 48th to 54th day, inclusive.

[3] One hundred milligrams of riboflavin given orally, followed by 50 mg next day, and a completely supplemented diet thereafter.

the morning exercise period. The D:N ratios on the third and 4th phlorhizin days averaged 3.38; those on the 5th and 6th, during thiamine therapy, averaged 3.42. Serum phosphatase increased markedly during phlorhizin administration.

TABLE 4

*Gluconeogenesis from protein during thiamine deficiency*

| TIME AFTER WITHDRAWAL OF THIAMINE | WEIGHT | FOOD CONSUMPTION | URINE NITROGEN PER DAY | URINE SUGAR PER DAY | D:N RATIO | SERUM PHOSPHATASE (BODANSKY, '33) |
|---|---|---|---|---|---|---|
| *days* | *kg* | *% of normal* | *gm* | *gm* | | *units* |
| | | | Dog 43 | | | |
| 3 | 12.85 | 100 | | | | |
| 6 | 12.70 | 100 | | | | |
| 9 | 12.66 | 100 | | | | |
| 12 | 12.47 | 100 | | | | |
| 13 | 12.43 | 100 | | | | 1.3 |
| 22 | 12.32 | 100 | | | | 0.7 |
| 25[1] | 12.14 | 100 | 12.62 | 46.95 | 3.72 | |
| 26 | 11.90 | 50 | 14.76 | 46.88 | 3.18 | |
| 27 | 11.79 | 0 | 11.67 | 41.48 | 3.56 | 7.4 |
| 28 | 11.11 | 0 | 14.22 | 45.45 | 3.19 | |
| 29[2] | 10.88 | 50 | 12.75 | 42.75 | 3.35 | |
| 30 | 10.88 | 50 | 9.80 | 34.20 | 3.49 | |
| 31 | 10.77 | 100 | 15.00 | 53.80 | 3.59 | 8.3 |
| | | | Dog 44 | | | |
| 3 | 14.88 | 100 | | | | 2.3 |
| 12 | 14.76 | 100 | | | | 3.0 |
| 21 | 14.68 | 100 | | | | 1.9 |
| 22[3] | 14.61 | 100 | 18.50 | 53.93 | 2.92 | |
| 23 | 14.27 | 0 | 14.50 | 49.43 | 3.40 | |
| 24 | 13.82 | 0 | 14.35 | 47.78 | 3.32 | |
| 25 | 13.48 | 0 | 13.20 | 42.30 | 3.20 | 5.0 |
| 26[4] | 13.24 | 50 | 12.60 | 45.80 | 3.64 | |
| 27 | 13.35 | 100 | 15.10 | 45.70 | 3.04 | |

[1] One gram phlorhizin injected twice daily for 7 days.

[2] One hundred milligrams of thiamine-HCl injected at 9 A.M. and again at 5 P.M. On the following day the complete vitamin supplement plus 50 mg of thiamine-HCl was added to the food.

[3] One gram of phlorhizin injected twice daily for 6 days.

[4] One hundred milligrams of thiamine-HCl injected at 8 A.M. and again at 5 P.M. The complete vitamin supplement was added to the diet.

Dog 44 likewise showed loss of weight, and anorexia appeared on the 22nd day of depletion. During the second, third, and 4th days of phlorhizin administration all food was refused, but the appetite returned promptly after thiamine was given. The D:N ratios on the third and 4th days of phlorhizin, during thiamine deficiency, averaged 3.26; those on the 5th and 6th days, during thiamine therapy, averaged 3.34. Serum phosphatase again showed an increase during administration of phlorhizin.

## DISCUSSION

It seems worthy of comment that thiamine deficiency appeared in our dogs in the usual three weeks although the diet was carbohydrate-free and contained over 72% of protein. On a similar diet, Dann ('45) maintained rats in excellent condition for more than a year, although stunting occurred.

Since the central nervous system disturbances noted in one of our dogs are not frequently seen during pyridoxine deficiency in this species, it should be stressed that the anemia characteristic of this deficiency did not develop in this animal, and that there was no hypoglycemia. Glucose and oxygen were therefore available, whether or not they were utilized normally. It is also of interest that Morgan, Groody and Axelrod ('46) observed pyridoxine deficiency in young dogs after 169 to 190 days of depletion when the diet contained 18.0% of casein. On a diet containing 45.8% of casein, deficiency appeared in 79 to 123 days. In our experiments on adult dogs receiving 72.2% of casein the time was still shorter. Morgan, Groody and Axelrod reported elevation of blood sugar and chlorides during pyridoxine deficiency. Elevation of blood sugar did not occur in our experiments, but our diet was free of carbohydrate. Serum chloride values, however, were all between 640 and 694 mg% as NaCl.

The lowest D:N ratios, observed in dog 38 during pyridoxine deficiency, would correspond to 44% of glucose from protein instead of 58%. Formation of glucose was therefore not seriously impaired even in this animal. However, the

consistent results in two animals with proved pyridoxine deficiency, together with theoretical considerations mentioned in the introduction to this paper, make the results as they relate to pyridoxine deficiency at least suggestive, particularly in the absence of similar findings in other deficiencies. The elevation of D:N ratios as riboflavin deficiency progressed in dog 40, and the diminution after termination of this deficiency in dog 43 (see text, page 98) are too small to be taken as evidence that oxidation of glucose formed from protein was impaired.

The observation that serum alkaline phosphatase activity increased during administration of phlorhizin is of considerable interest, and reference should be made to a number of pertinent studies. Marsh and Drabkin ('47) observed an increase in both acid and alkaline phosphatase activities of the kidney during alimentary hyperglycemia in rats. *In vivo* and *in vitro* inhibition of both acid and alkaline kidney phosphatase activities by phlorhizin was reported. Increases in both acid and alkaline liver phosphatase activities (Drabkin and Marsh, '47) and increases in serum alkaline phosphatase activity (Cantor, Tuba and Capsey, '47) have been reported in alloxan-diabetic rats. Treatment of these rats with insulin is reported to have restored the phosphatase activities to normal. The elevation of serum alkaline phosphatase which we observed in phlorhizinized dogs may be an adaptive response to inhibition of phosphatase activity by phlorhizin. It may also be related to the disturbance of carbohydrate metabolism in phlorhizin diabetes.

## SUMMARY

1. In dogs phlorhizinized during thiamine or riboflavin deficiencies of sufficient duration to produce the characteristic signs, formation of glucose from protein is not impaired. There is also no evidence that these deficiencies restrict utilization of glucose in the phlorhizinized animal.

2. In pyridoxine deficiency, the D:N ratios were quite low in one animal and somewhat low in another, and increased in

both animals following administration of the missing vitamin. The maximum change corresponded to formation of 44% instead of 58% of glucose from protein.

3. In dogs receiving a carbohydrate-free diet containing 72.2% of casein, pyridoxine deficiency appeared rapidly, and thiamine deficiency in the usual time, following withdrawal of the corresponding vitamin.

4. The disturbances in the central nervous system sometimes seen in dogs during pyridoxine deficiency were observed in one animal in the absence of hypoglycemia or anemia. The supply of glucose and oxygen to the nervous system is thus presumably normal, though the possibility of interference with utilization cannot be excluded.

5. Serum alkaline phosphatase was elevated during all phlorhizin periods in which it was determined. The relationship of this finding to changes in phosphatase observed in other forms of diabetes is discussed.

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# THE THREONINE REQUIREMENT OF THE CHICK

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TWO FIGURES

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Threonine has been reported to be a dietary essential for rats (Rose, '38), mice (Bauer and Berg, '43), and chicks (Hegsted, '44; Almquist and Grau, '44). In his amino acid mixture, Hegsted used 1.8% DL-threonine; Almquist and Grau used levels of 1%, 2% and 3%, and found that while there was no difference in growth rate between 2% and 3% levels, 1% appeared insufficient for growth. Grau ('47) obtained satisfactory growth with an amino acid mixture which contained 1.3% DL-threonine. On the basis of the threonine contents of various proteins and the growth rates observed when these proteins were fed to chicks, Almquist ('47) has estimated the requirement to be about 0.6% L-threonine. Wilkening, Schweigert, Pearson and Sherwood ('47) found that 0.67% L-threonine from gelatin and oxidized casein apparently satisfied the chick's requirements.

In the experiments reported here, the aim was to determine the threonine requirements more accurately. To this end, groups of birds were fed diets containing various levels of threonine in which the nitrogen source was a mixture of crystalline amino acids.

## METHODS

This report is based on the results obtained from 5 experiments performed over a two-year period. The amino acid

mixture used in the first experiment was identical with that used for the first experiment of a previously published report (Grau and Peterson, '46); a simpler mixture was used in subsequent experiments (table 1). The basal diet contained one

TABLE 1

*Mixtures of amino acids used in the basal diets*[1]

| AMINO ACID | LEVEL IN THE DIET | |
|---|---|---|
| | Expt. 1 | Expts. 2, 3, and 4 |
| | % | % |
| DL-Alanine | 1.5 | 1.5 |
| L-Arginine HCl | 1.4 | 1.4 |
| DL-Aspartic acid | 1.0 | .. |
| L-Cystine | 0.5 | 0.5 |
| L-Glutamic acid | 5.0 | 7.0 |
| Glycine | 1.8 | 1.8 |
| L-Histidine HCl $H_2O$ | 0.8 | 0.8 |
| L-Hydroxyproline | 0.2 | .. |
| DL-Isoleucine | 2.0 | 1.0 |
| L-Leucine | 2.0 | 1.5 |
| L-Lysine HCl $H_2O$ | 1.4 | 1.4 |
| DL-Methionine | 0 6 | 0.6 |
| DL-Norleucine | 0.2 | .. |
| DL-Phenylalanine | 1.5 | 1.0 |
| L-Proline | 2.0 | .. |
| L-Tryptophane | 0.3 | .. |
| DL-Tryptophane | | 0.6 |
| L-Tyrosine | 2.0 | 1.0 |
| DL-Valine | 2.0 | 1.5 |

[1] All amino acids were commercial products of C.P. grade.

of the amino acid mixtures of table 1 plus the following ingredients in grams per 100 gm of diet: cellulose [1] 5.0, crude soybean oil 5.0, calcium gluconate 8.0, tricalcium phosphate 2.0, sodium dihydrogen phosphate [2] 1.0, potassium chloride [3]

[1] Cellu flour.

[2] In some experiments, dipotassium phosphate was used instead of sodium dihydrogen phosphate; however, the levels of potassium, phosphorus and sodium were kept constant by manipulation of the potassium chloride level (see table 2).

[3] See footnote 2.

0.6, sodium bicarbonate 1.5, choline chloride [4] 0.2, inositol [5] 0.1, cholic acid [6] 0.1, mixed tocopherols [7] 0.05, and fortified sardine oil (3000 A–400 D per gm) 0.25; also in milligrams per 100 gm of diet, the following: thiamine hydrochloride 1, riboflavin 1, pyridoxine hydrochloride 1, nicotinic acid 5, calcium(d)pantothenate 3, 2-methyl-1,4-naphthohydroquinone diacetate 1, biotin [8] 0.1, pteroylglutamic acid [9] 1, manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8, and cobalt 0.5. DL-Threonine or L-threonine from commercial sources was added in varying amounts and glucose [10] was added to give a total of 100 gm.

In all experiments except number 2a a conditioning, or pre-test, diet was employed for a few days prior to the start of the experiments in order to accustom the chicks to the consistency of the experimental diets. The pre-test diet was essentially the same as the experimental diet except that 20% crude casein and 10% gelatin replaced the mixture of pure amino acids.

As will be pointed out in some detail, poor growth rates were obtained in experiments 2a and 3, even at levels of threonine which had allowed satisfactory growth in previous tests. For this reason, it appeared desirable to summarize all the variable experimental conditions in tabular form. These data are presented in table 2.

The growth rates are expressed as per cent gain per day,[11] in order to allow comparisons among experiments despite

[4] Provided by Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

[5] Omitted from the diets used in experiments 3 and 4 (see table 2).

[6] See footnote 5.

[7] Concentrate of natural mixed tocopherols (15%), Distillation Products, Inc.

[8] Provided by Merck and Co., through the courtesy of Dr. D. F. Green.

[9] See footnote 4.

[10] Cerelose.

[11] Per cent gain per day $= \frac{\text{Ave. gain during expt.} \times 100}{\text{Ave. weight during expt.} \times \text{no. days on expt.}}$.

slight differences in average starting weights and length of time of experiment. The actual gains can easily be calculated from the growth rates and the average weights at the start of the experiments (table 2).

TABLE 2

*Known variables which were present in the several experiments*

| EXP. NO. | NO. OF DAYS ON EACH DIET | | NO. OF CHICKS PER GROUP | WEIGHT AT START OF EXPERIMENT | | NO. OF DAYS ON EXPERIMENT | AMINO ACID MIXTURE USED [1] | BASAL DIET USED [2] | GROWTH RESULTS WITH POSITIVE CONTROL DIET |
|---|---|---|---|---|---|---|---|---|---|
| | Chick mash | Pre-test | | Average | Range | | | | |
| | | | | *gm* | *gm* | | | | |
| 1 | 14 | 3 | 4 | 93 | 85–100 | 10 | old | old | good |
| 2a | 14 | 0 | 4 | 77 | 75–79 | 8 | new | old | poor |
| 2b | 9 | 7 | 4 | 89 | 79–93 | 7 | new | old | good |
| 3 | 9 | 4 | 4 | 83 | 76–91 | 8 | new | new | poor |
| 4 | 9 | 5 | 3 | 92 | 86–98 | 7 | new | new | good |

[1] The differences between "old" and "new" amino acid mixtures are detailed in table 1.

[2] The "old" basal diet was exactly as presented under Methods; the "new" diet incorporated the changes stated in text footnotes 2 and 5, pages 106 and 107.

## RESULTS

### *Experiment 1*

Five groups of chicks fed diets which contained various levels of DL-threonine grew at the average rates shown in figure 1. The best growth rates observed here were as good as rates previously reported when chicks were fed mixtures of amino acids (Grau and Peterson, '46). From this experiment it was apparent: (1) that 0.5% DL-threonine is insufficient for satisfactory growth; and (2) that the requirement is certainly not much greater than 1% of the DL form.

### *Experiment 2a*

The best growth obtained with chicks fed these diets was only about 1.5% per day, which is considerably poorer than in experiment 1. Since these chicks were not fed the condition-

ing diet prior to the experiment, it was felt at the time that the chicks received a setback when the experimental diet was first presented to them. The point of inflection of the growth curve was at approximately 1% DL-threonine (fig. 1).

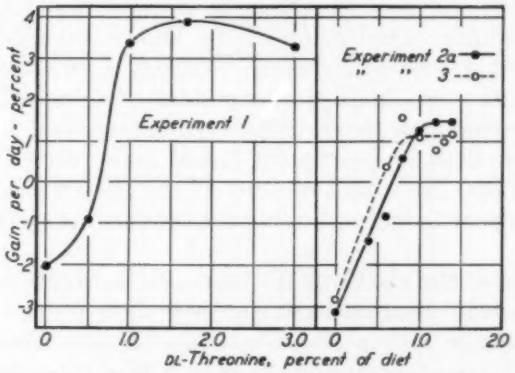


Fig. 1 The effect of feeding various levels of DL-threonine on the rate of growth. Each point represents the average growth rates of 4 chicks.

### *Experiment 2b*

Since the reason for the poor results obtained in experiment 2a was not apparent, the following experiment was performed: The unused diet of experiment 2a was weighed, mixed, and divided into two portions, to one of which 0.5% DL-isoleucine, 0.5% DL-valine and 0.2% DL-methionine were added. The DL-threonine content of both diets was 1.28%. After being fed a chick mash for 9 days and the pre-test diet for 7 days, chicks were sorted into two groups and given these diets. For the chicks receiving the extra isoleucine, valine and methionine, the growth rate was 3.9% per day; for those not receiving these supplements, the rate was 3.5% per day. The difference between these rates cannot be considered significant. Both compared very well with those obtained in experiment 1. It was apparent, then, that the amino acid mixture per se was not at fault, and the reason for the poor results of experiment 2a remained obscure.

*Experiment 3*

This experiment was essentially a repetition of experiment 2a, designed to test the reproducibility of the results of that experiment. The poor growth rates at high threonine levels, together with the good growth rates of experiment 2b, necessitated a critical examination of the known variables before experiment 4 was undertaken. Without a pre-test period, poor results were obtained in experiment 2a, but a three-day pre-test period appeared satisfactory in experiment 1. A 4-day period was used in experiment 3, and poor results were obtained. Thus, the length of time of the pre-test diet period did not appear to be responsible for the varying growth rates obtained.

The age of the chicks at the start of the experiments (the sum of days in columns 2 and 3 of table 2) is another variable which might be responsible. Good results were obtained with ages of 17 days (experiment 1) and 16 days (experiment 2b), and poor results were obtained with 14 days (experiment 2a) and 13 days (experiment 3). These age differences caused differences in average starting weights, also, as shown in columns 5 and 6 of table 2. These data, together with the fact that the amino acid mixture and basal diet used had no significant effect on the results obtained, led to the tentative hypothesis that the smaller chicks were not able to adjust to the amino acid diet, even though they had been fed a highly purified pre-test diet. As a test of this hypothesis the largest chicks available were chosen for experiment 4, but their age at the start of the experiment was only 14 days.

*Experiment 4*

Good growth was obtained at the higher levels of threonine, as shown in figure 2. In this figure the individual growth rate of each chick is presented, in order to allow a critical evaluation of the effectiveness of the natural enantiomorph of threonine as compared with DL-threonine. Here the abscissae are expressed in terms of L-threonine. If both D- and L-threonine

were effective in promoting growth, twice as much L-threonine as DL-threonine would be required to result in a particular rate of growth when this rate is below the maximum growth rate. The results of this experiment indicate that the L-threonine requirement is certainly not lower than 0.4% and not higher than 0.5% of the diet, and that the D form is not utilized by the chick to any significant extent.

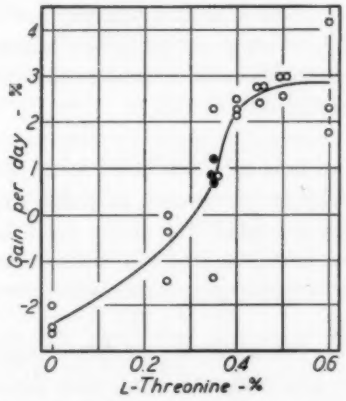


Fig. 2 The results of experiment 4. Each point represents a single chick. DL-Threonine was fed to all chicks denoted by open circles but only the L-threonine level is plotted here. The closed circles denote chicks fed L-threonine.

## DISCUSSION

The results presented above must be considered from several aspects before the tentative threonine requirements are set. The first concerns the validity of using mixtures of amino acids to determine quantitative requirements, in view of the acknowledged fact that the best growth rate obtained when a mixture is fed is less than the best one obtained when intact proteins are fed. Since there appears to be considerable justification for considering the amino acid data valid (Almquist, '47), tentative standards may be set up on this basis; as the studies of amino acid requirements are extended and refined, however, differences between whole and hydro-

lyzed proteins may become significant and further revisions of requirements may be needed.

It is apparent from the results plotted in figure 2 that only the natural, or L, form of threonine is utilized by the chick under these conditions; hence the data of figure 1 should be considered only in terms of the levels of L-threonine which were present. These data indicate that 0.5% L-threonine is adequate, and that 0.3% is inadequate, for best growth. Two of the three experiments which allow a close evaluation of the requirement indicate that 0.45% is as good as 0.5%; hence it seems justifiable to set the tentative L-threonine requirement of growing chicks at 0.45% of the diet.

As was stated above, the poor results observed in experiments 2a and 3 can best be correlated with the fact that comparatively small chicks were used in these experiments. The reason for the poor acceptability of the diets is not clear, since other experiments, which have given satisfactory results, have been performed with chicks comparable to the small chicks used here (Grau, '47).

According to recent analyses of the threonine content of feedstuff proteins (Schweigert, '48), only the proteins of bone meal and certain types of wheat contain insufficient threonine to satisfy the chick requirement. These calculations are based on the fact that a protein which is used to provide 20% protein to the diet must contain 2.25% threonine ($\frac{100 \times 0.45}{20}$) if the chick's requirement is to be met. Peanut meal protein is a marginal threonine source, but results of feeding trials have indicated that a 20% protein diet in which the sole amino acid source was peanut meal provided a satisfactory amount of threonine (Grau, '46).

## SUMMARY

Highly purified diets in which mixtures of amino acids replaced proteins were used to determine the level of threonine required for growth by young White Leghorn chicks. Four experiments were performed in which various levels of DL-threonine were fed; in one experiment, L-threonine was compared to DL-threonine for growth-promoting activity. DL-

Threonine was found to be only half as active as L-threonine; hence the requirement is best expressed in terms of the L-form. Under these conditions, the L-threonine requirement was found to be approximately 0.45% of the diet.

In two of the experiments poor growth was obtained even at satisfactory levels of threonine. Although the cause for these poor results could not be ascertained unequivocally from the data, it is suggested that the use of small chicks in some experiments was an important factor.

A comparison of the threonine requirement with the threonine content of feedstuff proteins indicates that a deficiency of this amino acid under practical conditions is unlikely.

ACKNOWLEDGMENT

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# THE EFFECT OF HEAT ON THE NUTRITIVE VALUE OF LACTALBUMIN

## I. GROWTH ON DIETS CONTAINING HEATED PROTEINS [1]

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The detrimental effect of heat on the nutritive value of proteins was first reported by McCollum and Davis, in 1917, and has since been the subject of continued investigation. The growth-promoting property of casein, white and whole wheat bread, rice, corn, and wheat gluten (Morgan, '31), edestin (Waisman and Elvehjem, '38) and soybean meal (Bird and Burkhardt, '43) has been shown to decrease when the protein is toasted or autoclaved under various conditions.

Greaves, Morgan and Loveen ('38) have reported that in casein, lysine is the first amino acid rendered nutritionally unavailable when the protein is heated, and that histidine is next in order. These authors observed much improved growth in rats when they supplemented diets containing heated casein with the two amino acids. Lysine supplementation has also been shown to improve the growth-promoting property of autoclaved edestin (Waisman and Elvehjem:, '38). On the other hand, Seegers and Mattill ('35), using beef protein, and Evans, McGinnis and St. John ('47), using soybean protein, have reported that heated protein is less susceptible to *in vitro* enzymatic digestion than is the unheated protein, and they have suggested that the alteration in digestibility

[1] The data in this paper are taken from the dissertation submitted by Ruth M. Davis for the degree of Doctor of Philosophy, Wayne University, 1948.

may be a major factor in the change in nutritive value of the proteins concerned.

The present report is based on a study of the nutritive value of heated lactalbumin with emphasis on the effect of varying the temperature used, the duration of the heating process, and the methods of heating. The value of lysine supplementation of diets containing heated lactalbumin, the effect of heat on the essential amino acids themselves, and the digestibility of heated lactalbumin have likewise been studied.

## EXPERIMENTAL

### *Plan*

Male albino rats approximately 25 days old and weighing between 40 and 60 gm were housed in individual cages with mesh bottoms designed to prevent coprophagy. Body weight changes and food intakes were recorded regularly. The basal diet contained 16.6 mg of nitrogen per gram of diet when lactalbumin was used (or 12–13.5% by weight of the heated protein), 51.2–49.7% dextrin, 12.8% sucrose, 20% corn oil,[2] and 4% salt mixture (Wesson, '32). The vitamin supplement incorporated into all the rations is shown in table 1.

The lactalbumin used in these experiments was a commercial product.[3] In some of the experiments it was baked in shallow layers approximately 5 mm deep at a constant temperature for one hour, and in other experiments the protein was autoclaved in similar layers at 116–120°C. under 15 lbs. pressure for 15, 30, or 60 minutes. The various heat treatments accorded the proteins are summarized in table 1. The color of the protein deepens progressively as the temperature increases when the protein is dry heated. However, when the protein is autoclaved at 120° for one hour the color is much darker than when it is dry heated at 140° for one hour, although not as dark as when it is dry heated at 200° for the same period. In fact, there appears to be a direct re-

[2] Mazola.

[3] Lactalbumin 15–42, obtained from the Borden Company, New York.

TABLE 1

*Diets: treatment of protein, and composition*

| TREATMENT OF PROTEIN | DESIGNATION OF DIET [1] | VITAMIN SUPPLEMENTS PER KG OF DIET | |
|---|---|---|---|
| Lactalbumin, unheated | UnL | Thiamine HCl [2] | 10 mg |
| Lactalbumin, dry heated for 1 hour at 140°C. | 140L–60D | Riboflavin [3] | 20 mg |
| | | Pyridoxine HCl [4] | 10 mg |
| | | Niacinamide [4] | 20 mg |
| Lactalbumin, dry heated for 1 hour at 120°C. | 120L–60D | Calcium pantothenate [4] | 40 mg |
| | | Choline [4] | 2000 mg |
| Lactalbumin, dry heated for 1 hour at 200°C. | 200L–60D | Inositol [4] | 2000 mg |
| | | *p*-Aminobenzoic acid [4] | 600 mg |
| Lactalbumin, autoclaved for 1 hour at 120°C. | 120L–60A | Biotin [4] | 10 μg |
| | | 2-Methylnaphthoquinone [4] | 4 mg |
| Lactalbumin, autoclaved for 30 min. at 120°C. | 120L–30A | α-Tocopherol [4] | 30 mg |
| | | Folic acid [5] | 20 mg |
| Lactalbumin, autoclaved for 15 min. at 120°C. | 120L–15A | Vitamin A [3,6] | |
| Gliadin, unheated | GL | Vitamin D [3,6] | |

[1] The abbreviations designating the diets refer to the protein component: "L" denotes lactalbumin; 120, 140, and 200 refer to the temperature used; 15, 30, and 60 refer to the duration of heating in minutes. The letter "D" shows that the protein was heated in a dry oven, while "A" represents the autoclaved protein.

[2] Courtesy of Parke, Davis and Company.

[3] Courtesy of Charles Pfizer and Company.

[4] Courtesy of Merck and Company.

[5] Courtesy of Lederle Laboratories.

[6] Four thousand units of vitamin A and 800 units of vitamin D were given per week, as Natola.

lationship between the color developed during the heating process and the nutritive value of the protein. Weast, Groody and Morgan ('48) observed similar color changes in their dry heated casein.

### *Growth*

Twenty rats were maintained on each of 4 dietary regimes: UnL, 120L–60A, 140L–60D, and 200L–60D (see footnote 1, table 1). Inasmuch as the animals given diet 200L–60D daily

TABLE 2

*Comparison of growth obtained under conditions of restricted food intake and ad libitum feeding*

| DIET [1] | | DAYS ON DIET | WEIGHT CHANGE | DAILY FOOD INTAKE | WEIGHT CHANGE PER GM FOOD EATEN |
|---|---|---|---|---|---|
| | | | *gm.* | *gm.* | *mg* |
| Average body weight changes — restricted intake | | | | | |
| UnL | (20)[2] | 21 | + 4.90 | 3.2 | + 59 |
| 140L–60D | (19) | 21 | + 2.77 | 3.2 | + 36 |
| 120L–60A | (20) | 21 | — 0.57 | 3.2 | — 10 |
| 200L–60D | (19) | 21 | — 15.45 | 3.2 | —233 |
| Average body weight changes — ad libitum | | | | | |
| UnL | ( 5) | 56 | + 132.4 | 7.8 | + 232 |
| 140L–60D | (10) | 56 | + 27.2 | 5.6 | + 69 |
| 120L–60A | ( 5) | 32 | — 10.4 | 5.1 | — 66 |
| 200L–60D | ( 5) | 18 | — 14.9 | 4.5 | —227 |

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

consumed the least food, the rats on the other diets were pair-fed with respect to this group. The animals given diet 200L–60D rapidly lost weight, whereas those given diet 120L–60A almost maintained their starting weight and those on diet 140L–60D were able to make a slight gain in weight, although they did not grow as well as did the control animals (table 2). When another series of animals was allowed the same diets ad libitum the differences in the growth-promoting abilities

of these various diets were more apparent but in the same order (table 2). Since the rats reacted to environmental conditions such as temperature and humidity, animals used during the summer did not grow as well as the others. Therefore control animals were used with the spring, summer, and fall groups and the experiments on diets 120L–60A, 140L–60D, 200L–60D were repeated at these periods, with essentially the same results.

*Amino acid supplementation*

Because of previous reports of the effectiveness of lysine and histidine in restoring the growth-promoting ability of heated proteins (Greaves, Morgan and Loveen, '38; Waisman and Elvehjem, '38), these amino acids were used as supplements with heated lactalbumin. Nine and six-tenths grams of lysine monohydrochloride were added to each kilogram of diet 200L–60D in preparing diet 200L–60D–Ly, and 3 gm of histidine monohydrochloride added to diet 200L–60D–Ly gave diet 200L–60D–LyH. Neither lysine nor lysine plus histidine restored the growth-promoting capacity of diets containing lactalbumin which had been baked at 200°C. for one hour (table 3).

The progressive nature of the changes produced in the protein by heating at various temperatures, as shown in table 2, suggested that lysine supplementation of lactalbumin might be efficacious with diets 120L–60A and 140L–60D. Table 3 shows that growth rates of the rats fed lysine supplements did not approach those of the control animals. Rats that had been on a restricted intake of diets UnL, 140–60D, or 120L–60A for three weeks were allowed to eat ad libitum for the following 8 weeks. One animal on the UnL diet, three rats on diet 120L–60A, and 4 rats on diet 140L–60D received the same diet that they had received for the first three weeks. Four others previously on diet 120L–60A and 4 on diet 140L–60D were given the lysine supplement (9.6 gm per kilogram of diet). These animals consumed more food and gained more weight than rats fed similar diets from weaning and

without the prior restricted intake period. However, the lysine supplement was only slightly beneficial with diet 140L–60D and of no value with diet 120L–60A. The control animal exhibited the same acceleration in growth rate during this period as was seen in the others but gained significantly more weight per gram of food consumed than the rats fed

TABLE 3

*Effect of supplementing heated lactalbumin and gliadin with lysine, or histine, or both*

Average body weight changes

| DIET [1] | DAYS ON DIET | WEIGHT CHANGE | DAILY FOOD INTAKE | WEIGHT CHANGE PER GM FOOD EATEN |
|---|---|---|---|---|
| | | *gm* | *gm* | *mg* |
| 200L–60D (15)[2] | 21 | — 15.8 | 3.8 | —193 |
| 200L–60D–Ly ( 5) | 21 | — 15.4 | 3.7 | —199 |
| 200L–60D–LyH ( 5) | 21 | — 14.7 | 3.8 | —183 |
| 140L–60D ( 4) | 56 | + 82.9 | 8.1 | +180 |
| 140L–60D–Ly ( 4) | 56 | +106.6 | 8.4 | +224 |
| 120L–60A ( 3) | 56 | + 35.3 | 5.0 | +118 |
| 120L–60A–Ly ( 4) | 56 | + 32.3 | 4.7 | +122 |
| UnL ( 1) | 56 | +188.0 | 9.3 | +355 |
| 140L–60D (10) | 56 | + 27.1 | 6.4 | + 69 |
| 140L–60D–3Ly (10) | 56 | + 23.0 | 6.0 | + 59 |
| UnL | 56 | +132.4 | 7.8 | +232 |
| GL (10) | 56 | — 9.4 | 4.1 | — 41 |
| Gl–Ly (10) | 56 | + 18.2 | 5.1 | + 60 |
| Gl–140Ly (10) | 56 | + 17.8 | 5.5 | + 57 |

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

the heated protein. To insure adequate supplementation the amount of lysine used in diet 140L–60D–Ly was tripled and another series of rats was given this diet (140L–60D–3Ly). The supplementation produced no improvement in the growth of these animals (table 3) and it can be concluded that the decrease in the nutritive value of heated lactalbumin is not primarily due to the unavailability of lysine to the animal.

Further evidence that heat does not injure the lysine fraction per se was obtained by heating lysine monohydrochloride at 140°C. for one hour in a dry oven and comparing the ability of the heated (140Ly) and unheated (Ly) amino acid to promote growth in rats maintained on diets in which gliadin was the sole source of protein (table 3). No differences were observed in the growth rates of the animals receiving the two supplements; the rats apparently were able to utilize the heated as efficiently as the unheated amino acid.

TABLE 4

*Growth on diets with and without heated amino acids*

Average body weight changes

| DIET | DAYS ON DIET | WEIGHT CHANGE | DAILY FOOD INTAKE | WEIGHT CHANGE PER GM FOOD EATEN |
|---|---|---|---|---|
| | | *gm.* | *gm.* | *mg* |
| AAH (10)[1] | 35 | + 53.0 | 8.1 | + 188 |
| AA ( 8)[2] | 35 | + 44.0 | 7.7 | + 167 |

[1] Figures in parentheses denote number of rats used.
AAH signifies amino acid mixture, heated.

[2] Signifies amino acid mixture, unheated.

Greenhut et al. ('48) have shown that there is no loss of lysine, threonine or methionine in the process of roasting meat. However, there have been no reports in the literature upon the effect of heat on a mixture of the 10 essential amino acids; therefore, such a mixture [4] was prepared (according to Rose, '38) and heated dry at 140°C. in an oven for one hour. The mixture, both heated (AAH) and unheated (AA), constituted 12% of the total diet. Under these conditions none of the essential amino acids was so changed by heat as to render it unavailable to the animal for growth, as compared with the growth obtained by the rats fed the same mixture unheated (table 4). In contrast, the nutritive value of lactalbumin is considerably decreased by such treatment.

[4] Courtesy of Frederick Stearns and Company.

*Digestibility*

Observations on the animals fed diet 200L–60D suggested that these animals received no benefit from the protein component of the ration. To test this theory, 5 rats were fed a diet containing no protein (diet NP) but otherwise identical with diet 200L–60D, with dextrin replacing the protein isocalorically. The rats on diet NP lost almost the same amount of weight as those on diet 200L–60D (15.4 gm and 15.8 gm, respectively) and at the same rate, over a three-week period, and consumed 3.7 gm of diet daily as compared to the 3.8 gm daily consumed by the 200L–60D group. The weight change per gram of food consumed by rats on diets NP and 200L–60D was —206 and —202 mg, respectively.

In order to ascertain whether the lowered nutritive value of the heated protein was the result of decreased digestion and/or absorption, or whether it resulted from non-utilization of the absorbed amino acid, a comparative study was made of the fecal nitrogen excretion of rats maintained on diets 140L–60D, 120L–60A, NP, UnL, and Tox (diet UnL to which 60 gm of lactalbumin which had been heated at 200°C. for one hour was added per kilogram of diet). Feces were collected from individual rats over 24-hour periods after the animals had consumed their respective rations for at least one week, and the total 24-hour collection was analyzed for total nitrogen. The average amount of fecal nitrogen excreted by the rats on diet NP was considered the basal, or endogenous, nitrogen, and this quantity was subtracted from the amount of nitrogen excreted by the animals on protein-containing diets. The rats on diet NP excreted 2.33 mg of nitrogen per gram of food consumed, or an average of 8.3 mg of nitrogen per 24 hours. The fecal nitrogen excretion of the rats on all the diets containing heated protein was higher than was that of the control animals (table 5).

The suggestion that the lowered nutritive value of heated lactalbumin was the result of a decreased digestibility of the protein was further supported by an *in vitro* study. A quan-

tity of the protein equivalent to 0.55 gm of nitrogen was incubated with pepsin (pH 1.5) and then pancreatin (pH 8.0) at 37°C. for a total of 7 days, and the mg of nitrogen present in an aliqout of the supernatant fluid determined by Sorenson's formol titration procedure. As can be seen in table 5, the amount of digestion occurring *in vitro* correlates well with the ability of the rats to grow on the various diets. It appears, therefore, that the change in the digestibility of the protein after heating is a significant factor in the decrease in its nutritive value.

TABLE 5

*Digestibility of heated protein — in vivo and in vitro observations*

| IN VIVO | | IN VITRO | |
|---|---|---|---|
| Diet [1] | N excreted per gm food consumed | Protein | N in supernatant fluid after in vitro digestion |
| | *mg* | | *mg %* |
| UnL (10)[2] | 2.49 ± 0.24 [3] | UnL | 120.0 |
| 140L–60D (10) | 7.77 ± 0.83 | 140L–60D | 82.9 |
| 120L–60A (8) | 6.18 ± 0.97 | 120L–60A | 34.7 |
| Tox (10) | 8.43 ± 0.90 | 200L–60D | 4.1 |

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

[3] Standard error.

### *Methods of heating*

In addition to varying the temperature used during the heating process, the effect of varying the methods of heating and the duration of heating was studied. Rats maintained on diets containing lactalbumin which had been autoclaved at 120°C. (15 lb. pressure) for one hour (diet 120L–60A) lost an average of 10.4 gm in 32 days, whereas those animals fed lactalbumin which had been oven-baked at 120°C. for one hour (diet 120L–60D) gained an average of 56.8 gm in that period. Although the rats on diet 120L–60D gained slightly less weight than did the controls (56.8 gm and 68.4 gm respectively), it is obvious that the decrease in the nutritive value of lactalbumin is considerably greater when the protein

has been autoclaved at 120°C. than when it has been heated in a dry oven at the same temperature. The average daily food intakes of the animals fed diets 120L–60A, 120L–60D, and UnL were 5.1 gm, 9.5 gm, and 9.6 gm respectively, and their respective weight changes per gram of food consumed were −66 mg, + 187 mg and + 223 mg.

Table 6 shows that autoclaving at the same temperature and pressure (120°, 15 lb.) for 15 or 30 minutes (Diets 120L–15A and 120L–30A) reduces the nutritive value of lactalbumin

TABLE 6

*Influence of duration of heating*

Average changes in body weight

| DIET [1] | DAYS ON DIET | WEIGHT CHANGE | DAILY FOOD INTAKE | WEIGHT CHANGE PER GM FOOD EATEN |
|---|---|---|---|---|
| | | *gm* | *gm* | *mg* |
| UnL ( 5)[2] | 56 | + 132.4 | 7.8 | + 232 |
| 120L–15A ( 9) | 56 | + 41.5 | 7.4 | + 100 |
| 120L–30A (10) | 56 | + 11.3 | 6.6 | + 25 |
| 120L–60A ( 5) | 32 | — 10.4 | 5.1 | — 66 |

[1] For meaning of symbols, see table 1.

[2] Numbers in parentheses denote number of rats used.

to a considerable degree. Because of the wide use of the pressure cooker in the American household, the authors believe that these findings warrant further investigation.

The animals used in the various experiments were autopsied and complete histological examinations were made. The results will be reported in a later communication.

## DISCUSSION

The data presented above show that the nutritive value of lactalbumin is decreased progressively when the protein is heated. The temperature, duration of heating, and method of heating all are factors which determine the amount of damage sustained by the protein. Pressure and/or moisture

accelerates the production of the deleterious change which occurs in lactalbumin when it is heated.

Contrary to the results obtained with casein by Morgan ('31) and with edestin by Waisman and Elvehjem ('38), the addition of lysine to diets containing heated lactalbumin did not significantly improve their biological value, and it can be concluded that the decreased nutritive value of lactalbumin after heating is not primarily the result of a change in the lysine fraction. There was no evidence of a toxic factor in the heated protein. The change produced in this protein by heat does considerably decrease the digestibility of the protein, to the extent that when the protein has been heated at 200°C. for one hour it is practically indigestible. Although Greaves, Morgan and Loveen ('38) using rats as the experimental animal, reported that the decrease in the digestibility of heated casein was not sufficient to explain the decrease in the biological value of the same protein, this does not appear to be the case with heated lactalbumin. Evans, McGinnis and St. John ('47), using chicks as the experimental animal, have found that the digestibility of over-cooked soybean meal is also decreased. In a recent communication Weast, Groody and Morgan ('48) reported that as much as 94–96% of the nitrogen excreted by dogs fed casein heated at 200° appeared in the feces. We have found that the nitrogen of heated lactalbumin is similarly not absorbed by the rat.

In view of the fact that heating (140°C.) the indispensable amino acids does not decrease their utilization, as shown by comparative growth studies, the change produced in a protein by heat probably does not affect the structure of the amino acid per se. The site of the alteration in heated lactalbumin appears to be at the linkages between the amino acids; either the peptide linkages or the grid linkages or both are so changed by heat as to become resistant to enzymatic hydrolysis. The progressive nature of this change may indicate that certain linkages are more susceptible to change by heating than others.

SUMMARY

The nutritive value of lactalbumin is diminished by heating the protein. This decrease appears to be the result of lowered digestibility of the heated protein due to a change in the linkages between the amino acids which renders them less susceptible to enzymatic hydrolysis. This change is produced at lower temperatures and in less time when the protein is autoclaved than when it is baked in a dry oven.

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# THE EFFECT OF PTEROYLGLUTAMIC ACID AND AN UNIDENTIFIED FACTOR ON EGG PRODUCTION AND HATCHABILITY[1]

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Bauernfeind and Norris ('39) and Schumacher, Heuser and Norris ('40) reported a factor necessary for breeding hens which they designated Factor R and which subsequently has been suggested to be a mixture of folic acid conjugates (Charkey, Daniel, Farmer, Norris and Heuser, '47). The essential nature of a factor present in the *Lactobacillus casei* ε eluate fraction of solubilized liver extract for breeding hens was established by Cravens, Sebesta, Halpin and Hart ('42). The identification of the *L. casei* factor as pteroylglutamic acid was established by Angier et al. ('46). Since synthetic pteroylglutamic acid (PGA) has become available, Taylor ('47) and Schweigert, German, Pearson and Sherwood ('48) have published reports on the quantitative needs of the breeding hen for this vitamin. Taylor ('47) reported that 0.12 mg per kilogram of ration was essential for egg production, while an amount somewhat in excess of this was required for satisfactory hatchability. Schweigert et al. ('48), on the other hand, reported that the requirement is between 0.12 and 0.42 mg per kilogram of ration.

During the course of studies designed to determine the effect of pteroylglutamic acid on egg production and hatch-

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ability it became obvious that the results were complicated by a deficiency of an unidentified factor or factors. Data will be presented in this report indicating the importance of such a factor or factors for hatchability. Further, some of the data may shed some light on the quantitative needs of the breeding hen for pteroylglutamic acid.

## EXPERIMENTAL

Single Comb White Leghorn pullets were used in all experiments. The birds were housed in individual-cage laying-batteries with raised screened floors. They were artificially

TABLE 1

*Composition of experimental diets*

| INGREDIENTS | BASAL DIETS | | | | VITAMINS, CHOLINE AND INOSITOL ADDED TO ALL BASAL DIETS | |
|---|---|---|---|---|---|---|
| | B20 | B22 | B23 | B24 | | |
| | % | % | % | % | | *mg/kg of diet* |
| Dextrin | 64 | 67 | .. | .. | Riboflavin | 6.0 |
| Sucrose | .. | .. | 67 | 67 | Thiamine-HCl | 4.0 |
| Casein [1] | 18 | 18 | 18 | 18 | Ca pantothenate | 15.0 |
| Gelatin | 5 | 5 | 5 | 5 | Niacin | 100 |
| Salts IV | 5 | 5 | 5 | 5 | Pyridoxine-HCl | 4.0 |
| Soybean oil | 3 | 3 | 3 | 3 | 2-Methyl 1,4-naphthoquinone | 0.5 |
| Vitamins A and D (400D–3000A) | 2 | 2 | 2 | 2 | Biotin | 0.2 |
| Liver fraction L | 3.0 | .. | .. | .. | Alpha-tocopherol | 3.0 |
| Fish solubles (50% solids) | .. | .. | .. | 3.0 | Choline | 2,000 |
| | | | | | Inositol | 1,000 |

[1] Vitamin test. General Biochemicals, Inc.

inseminated weekly with pooled semen from New Hampshire males. All birds were weighed weekly. Eggs were collected daily, marked with the hen's number and recorded. Settings of eggs were made weekly. All eggs failing to hatch were broken to determine fertility and to make gross examinations of the embryos for developmental abnormalities.

The composition of the basal diets used is given in table 1. Feed, water and oyster shell were supplied ad libitum.

Experiment 1 was designed to obtain information on the pteroylglutamic acid requirements of the breeding chicken. Five groups of 4 birds each were fed the following rations for 11 weeks: Group 1, diet B20 (which served as a control since this diet had proved satisfactory in previous experiments); group 2, diet B22; groups 3–5, ration B22 with pteroylglutamic acid additions at levels of 0.1, 0.25 and 1.0 mg per kilogram of diet, respectively. The hatchability and egg production data are presented in table 2.

Experiment 2 was similar in design to experiment 1. The following diets were fed to 6 groups of three birds each for 14 weeks: Group 1, diet B20; group 2, B22; and groups 3–6,

TABLE 2

*Effect of pteroylglutamic acid on egg production and hatchability*

| PGA SUPPLEMENT TO DIET B22: | EXPERIMENT 1 | | EXPERIMENT 2 | |
|---|---|---|---|---|
| | Egg production | Hatchability | Egg production | Hatchability |
| *mg/kg* | % | % | % | % |
| 0 | 54 | 40 | 36 | 32 |
| 0.10 | 59 | 56 | 40 | 35 |
| 0.20 | | | 39 | 28 |
| 0.25 | 61 | 51 | | |
| 0.50 | | | 31 | 60 |
| 1.0 | 51 | 66 | 51 | 20 |
| 3% Liver L | 57 | 83 | 55 | 71 |

diet B22 supplemented with 0.1, 0.2, 0.5 and 1.0 mg of pteroylglutamic acid per kilogram of diet, respectively. The hatchability and egg production data are given in table 2.

It is obvious from the results shown in table 2 that none of the diets fed in experiments 1 and 2, except perhaps the control, was adequate for good hatchability. It is also obvious that the response of the birds to the same diets in the two experiments was radically different. The possible significance of this observation will be discussed later, but suffice it to point out here that the results of experiments 1 and 2 show definitely that some unidentified factor, or factors, needed by breeding hens is missing from diet B22 supplemented with

PGA. It was also suspected that intestinal synthesis on the dextrin rations used in these experiments might be one factor responsible for certain of the erratic results observed, since Couch et al. ('48a) have shown that dextrin promotes intestinal synthesis of biotin in the mature fowl. Thus an experiment was designed to take these factors into consideration.

In the third experiment sucrose was compared with dextrin as a carbohydrate source and condensed fish solubles was tested as a source of unidentified factors. Additional purified casein was also added to eliminate the possibility that insufficient protein or amino acids might be affecting the results. The diets fed and the resulting egg production and hatchability for experiment 3 may be seen in table 3.

TABLE 3

*Rations fed and per cent of egg production and hatchability (Experiment 3)*

| GROUP [1] | BASAL DIET | SUPPLEMENT TO BASAL DIET | EGG PRODUCTION | HATCHABILITY | | |
|---|---|---|---|---|---|---|
| | | | | Start of exp. | End of exp. | Ave. |
| | | | % | % | % | % |
| 1 | B22 | None | 59 | 75 | 29 | 62 |
| 2 | B23 | None | 36 | 90 | 0.0 | 37 |
| 3 | B22 | 4.0 mg per kg PGA | 62 | 100 | 40 | 72 |
| 4 | B23 | 4.0 mg per kg PGA | [2] | 74 | 8.0 | 65 |
| 5 | B22 | 6% casein [3] | 51 | 87 | 17 | 45 |
| 6 | B22 | 6% casein plus 4.0 mg per kg PGA | 79 | 100 | 29 | 73 |
| 7 | B23 | 6% casein plus 4.0 mg per kg PGA | 61 | 96 | 20 | 76 |
| 8 | B22 | 3% liver fraction L | 82 | 75 | 91 | 83 |
| 9 | B23 | 3% liver fraction L | 75 | 89 | 88 | 84 |
| 10 | B23 | 3% liver fraction L plus 6% casein | 76 | 74 | 87 | 94 |
| 11 | B22 | 4.0 mg per kg PGA plus 3% fish solubles | 67 | 75 | 90 | 92 |
| 12 | B23 | same as group 11 | 76 | 90 | 71 | 86 |
| 13 | Practical ration | | 68 | 95 | 95 | 93 |

[1] Four birds in each group.

[2] Not presented because of loss of two birds in this group.

[3] Vitamin test casein. General Biochemicals, Inc.

From the data shown in this table it appeared that satisfactory egg production and hatchability could be obtained using either sucrose or dextrin as a source of carbohydrate when liver fraction L or fish solubles and 4.0 mg of pteroylglutamic acid per kilogram of ration were included in the diets. With the results of experiment 3 at hand it appeared that a diet was available (group 12 of experiment 3) which

TABLE 4

*Rations fed and per cent of egg production and hatchability (Experiment 4)*

| GROUP [1] | BASAL DIET | SUPPLEMENT TO BASAL DIET | EGG PRODUCTION | HATCHABILITY | | |
|---|---|---|---|---|---|---|
| | | | | Start of exp. | End of exp. | Ave. |
| | | | % | % | % | % |
| 2 | B23 | 4.0 mg per kg PGA | 48.5 | 75 | 0 | 27 |
| 3 | B22 | Same as group 2 | 70.8 | 86 | 36 | 54 |
| 4 | B24 | None | 50.9 | 89 | 0 | 26 |
| 5 | B24 | 0.25 mg per kg PGA | 74.7 | 90 | 0 | 46 |
| 6 | B24 | 0.50 mg per kg PGA | 70.5 | 95 | 0 | 65 |
| 7 | B24 | 0.75 mg per kg PGA | 73.8 | 100 | 0 | 53 |
| 8 | B24 | 1.0 mg per kg PGA | 69.9 | 89 | 0 | 75 |
| 9 | B24 | 4.0 mg per kg PGA | 62.8 | 100 | 40 | 76 |
| 10 | B22 | 3% fish solubles | 55.7 | 87 | 31 | 53 |
| 11 | B22 | Same as group 10 plus 4.0 mg per kg PGA | 59.5 | 95 | 36 | 80 |
| 12 | B23 less gelatin | Same as group 2 | 43.9 | 100 | 0 | 41 |
| 13 | Practical control | | 55.1 | 100 | 67 | 96 |

[1] Four birds in each group.

would make possible studies on the quantitative needs of breeding chickens for pteroylglutamic acid. Experiment 4 was then set up to gain evidence on this point and to obtain further evidence on the unidentified factor as well as the possible effect of the source of carbohydrate on the reaction of the birds to the diets used. The diets fed and the egg production and hatchability data for experiment 4 are given in table 4.

## RESULTS AND DISCUSSION

### *Unidentified factors*

The results of experiments 1 and 2, given in table 2, are of interest because they emphasize the need of breeding hens for some unidentified factor which is essential for embryonic development. Further, the data indicate that the bird may be depleted of this factor in a relatively short period of time. It was mentioned previously that a marked difference in response of the birds to identical rations in experiments 1 and 2 was observed. In experiment 2 the birds depleted much more quickly and uniformly than in experiment 1. In experiment 2 hatchability of all groups dropped to zero by the 7th week, while in experiment 1 only two groups dropped to zero by the 6th week and even in these hatchability again improved as the experiment progressed.

Data which suggest that diets containing dextrin may promote the synthesis of an unidentified factor or factors in the intestinal tract are given in tables 3 and 4. A comparison of the hatchability of the eggs from groups 3 and 4 of experiment 3 (table 3) shows that those of group 3 which were produced by hens receiving dextrin as a carbohydrate hatched better than those from hens receiving sucrose as a carbohydrate. The results of experiment 4, given in table 4, further indicate that dextrin stimulates the intestinal synthesis of an unidentified factor or factors essential for the breeding hen. The hens of group 3, which received dextrin as a carbohydrate, produced eggs which hatched significantly better than those from hens of group 2, which were receiving sucrose as a carbohydrate.

Further evidence showing the importance in the diet of breeding hens of an unidentified factor in fish solubles and liver fraction L is given in the results of experiments 3 and 4, presented in tables 3 and 4. In every case a rapid decline (to less than 20% in 4 weeks) in hatchability occurred when a sucrose diet containing no fish solubles or liver fraction L, but containing 4.0 mg per kilogram of pteroylglutamic acid

was fed to the birds. The drop in hatchability was prevented by the addition of either fish solubles or liver fraction L. Two birds of group 4 were discarded at 4 weeks as they had lost considerable weight and had developed anorexia. The injection of 0.1 ml of reticulogen [2] brought about a dramatic recovery, but obviously the two birds had to be eliminated from the group.

The addition of casein did not improve hatchability. It is interesting to note, however, that the addition of 6% purified casein (group 5) appeared to result in a more rapid depletion of the birds (to 13% in 4 weeks) and that this effect was apparently overcome by the addition of pteroylglutamic acid (group 6). The birds of group 12 which received the same diet as group 2, except that gelatin was omitted, did not deplete any more rapidly than those of group 2.

There is some evidence from these experiments that a deficiency of the factor found in fish solubles and liver fraction L resulted in a decline in egg production. For example, group 4 of experiment 3 produced eggs at a very low rate and, as mentioned previously, two of the birds in this group showed a pronounced decline in weight. By comparing the rate of production of the groups of birds receiving dextrin and no fish solubles with that of the groups receiving a similar diet containing sucrose (tables 3 and 4), it may be seen that the dextrin-fed birds produced at a higher rate, as would be expected if intestinal synthesis made available to the bird a limited quantity of the factor yet one sufficient to support egg production. It is believed, however, that additional data are necessary to demonstrate the possible effect of this factor on egg production, since with such great individual variations, the numbers involved in the present experiments are inadequate to determine significance.

The studies reported herein dealing with an unidentified factor or factors essential for the breeding bird are of interest chiefly because they demonstrate that the laying bird can be depleted very quickly of an unidentified factor es-

[2] Lilly.

sential for hatchability. Numerous reports dealing with unidentified factors essential for breeding hens have appeared, including those of McGinnis et al. ('44), Bird et al. ('46), Bethke et al. ('46), and Cravens et al. ('46). Rubin et al. ('47) presented data which indicated that 15–16 weeks of feeding all-vegetable diets deficient in an unidentified factor were required to deplete the birds. Rubin, Bird and Rothchild ('46) suggested that intestinal synthesis of an unidentified factor might occur in the mature bird since hen feces were found to be a good source of unidentified chick growth factors essential in all-vegetable chick rations. McGinnis, Stevens and Groves ('47) presented evidence which they interpreted as indicating that the growth factors were synthesized, for the most part, by microorganisms after the feces were voided and not in the intestinal tract of the hen. Similar results have been reported by Kennard et al. ('48). The data presented herein are in agreement with the work of Rubin, Bird and Rothchild ('46), and these data further suggest that such synthesis may be, in part, responsible for the prolonged depletion period required when diets are employed which promote intestinal synthesis. It is probable, however, that other unrecognized conditions affect the rate of depletion since, even with the purified diets employed in the present study, considerable variation in degree and time of depletion was observed. It seems logical to conclude, nevertheless, that rapid depletion may be obtained if conditions are right, since there is abundant evidence that the hen may be quickly depleted of water soluble vitamins, and the unidentified factor, or factors, from cow manure, fish solubles, fish meal and certain liver fractions is water soluble.

### *Pteroylglutamic acid*

The results of experiment 4, given in table 4, fail to provide clear-cut data on the pteroylglutamic acid requirements of the breeding hen. It would appear from these data that between 0.5–1.0 mg per kilogram of diet is essential for good hatchability, but even at a level of 4.0 mg per kilogram hatchability was not maintained at a high level for the 12-week

experiment. A rapid falling off in hatchability had occurred on the 0.25, 0.50 and 0.75 mg levels by the 9th week of the experiment, while a similar fall in hatchability occurred at the 10th and 11th weeks for the 1.0 and 4.0 mg levels, respectively. The significance of these results is not apparent at this time and the possibility exists that the fall in hatchability at 11 and 12 weeks is related to some environmental conditions, since the control group (13) dropped to 67% during the last week of the experiment. Of course, the possibility also exists that the birds had become depleted of a second factor by this time, or that 3% fish solubles supplied inadequate quantities of the factor to support hatchability. It is obvious, however, that further experiments are necessary to determine the reason for the pronounced drop in hatchability observed during the latter phases of experiment 4.

There is no question but that the birds in group 4, experiment 4, produced eggs at a significantly lower rate than did the birds receiving an adequate amount of pteroylglutamic acid. The average production of the birds of group 4, given in table 4, does not give a true picture of the egg production data of this group, since there was a gradual decline in egg production to 11% by the end of the experiment. The drop in production was most rapid after the 8th week. No such decline was observed in group 5, which received 0.25 mg of pteroylglutamic acid per kilogram of diet. Neither was there a pronounced decline in the production of group 10, which was fed a diet deficient in pteroylglutamic acid with dextrin as the carbohydrate. These results suggest that 0.25 mg of this vitamin per kilogram of ration is adequate for egg production and, further, that with the rations containing carbohydrates that promote intestinal synthesis pteroylglutamic acid may be synthesized in sufficient quantities to meet the needs of the laying bird.

The results of this study are not in agreement with the results reported by Schweigert et al. ('48) regarding the pteroylglutamic acid requirements of the breeding bird. It appears likely that the higher requirement indicated in the

present study (0.5–1.0 mg per kilogram) results from the use of a type of basal diet which does not favor intestinal synthesis of the vitamin. That intestinal synthesis was a factor affecting hatchability may be seen by comparing the results of groups 4 (sucrose) and 10 (dextrin), table 4. Hatchability of eggs from the birds of group 10 declined rapidly, but after an apparent adjustment period it again increased, only to decline again. Such variations have been noted in studies on the effects of various carbohydrate sources on the intestinal synthesis of biotin (Couch et al., '48b). The diets employed by Schweigert et al. would appear to favor such synthesis and thus might lead to the suggestion of requirements far below the true optimum.

## SUMMARY

1. Four experiments with breeding chickens have been described which demonstrate the importance for hatchability of an unidentified factor or factors.

2. Fish solubles and liver fraction L are good sources of the factor or factors.

3. The breeding hen may be depleted of the factor in from 4–6 weeks when purified diets are used.

4. Data are presented which indicate that dextrin favors the intestinal synthesis of the factor in the mature fowl.

5. The pteroylglutamic acid requirement of the laying bird is probably not over 0.25 mg per kilogram of diet.

6. The pteroylglutamic acid requirement of the breeding bird appears to be approximately 0.5–1.0 mg per kilogram of diet when a diet is employed which does not favor intestinal synthesis of the vitamin.

7. Evidence is presented which indicates that pteroylglutamic acid synthesis in the intestinal tract of the breeding hen is favored by diets containing dextrin as a carbohydrate.

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# COMPARISON OF CHEMICAL ANALYSIS AND BIO-ASSAY AS MEASURES OF VITAMIN A VALUE OF SOME VEGETABLES AND THE EFFECT OF COMMINUTION UPON THE BIO-ASSAY VALUE

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Vegetables and fruits have been estimated to supply about two-thirds of the vitamin A value of the food consumed in the United States (Clark, Friend and Burk, '47); the green leafy and yellow vegetables (including sweet potatoes), which alone furnish about half of the total, are the most important single group of contributors. These calculations were based on figures in which weight was given to chemical measurements of carotene content as well as to biological measurements of vitamin A value reported in the literature. However, the carotene content of certain vegetables and fruits, particularly the yellow ones, measured by chemical means, has been shown to be an unreliable index of their actual vitamin A value. Graves ('42) says that when the results are to be "related to nutritional problems, the danger of relying on carotene estimations even as an index of the comparative activity of the yellow and orange vegetables can hardly be over-estimated."

Conversion factors for different vegetables, calculated from strictly parallel chemical and biological assays, would make it

possible to adapt results of the more economical chemical measurements of carotene content for use in dietary calculations. Such assays are also necessary to ascertain what factors other than the nature of the vegetable itself may influence, either favorably or adversely, the availability of carotene. Smith and Otis ('41, '43) demonstrated that there is great variation in both rat growth and vitamin A liver stores promoted by the same amount of carotene, depending upon the vegetable source, and observed that "yellow foods are especially inefficient in promoting vitamin A reserves." Their data, however, do not lend themselves to quantitative interpretation because of the methods employed. According to experiments reported by Fraps and Meinke ('45), differences exist between vegetables and carotene in oil solution and among the vegetables themselves with respect to the availability of the carotene for accumulation of vitamin A in the liver; Kemmerer and Fraps ('45) were unable to find such differences, however, when the suboptimum rat-growth rate employed in bioassays was the criterion. Wilson et al. ('42, '46) have reported good agreement between chemical and rat growth assays of the vitamin A value of fresh carrots and of fresh sweet potatoes, but the number of animals used was too small for reliable interpretation of the bioassay results.

No account is given in any of these papers of the technics employed to assure that the sample of the vegetable analyzed chemically was as representative as possible of the small portions received by the rats as supplements. Discrepancies between chemical analyses and bioassays have frequently been attributed to sampling errors (Bacharach, '41). Kemmerer and Fraps ('45) chromatographed the plant pigments and calculated their β-carotene equivalent, so that the second inaccuracy commonly encountered in measurements of carotene content, i.e., inadequate differentiation among the carotenoids, was eliminated as a source of error in their studies.

Orent-Keiles, Callison, Schaevitz and Frenchman ('46) have published results of experiments comparing the chemical and biological assay of carotene in cooked kale and also com-

paring the carotene so measured with the carotene after extraction from the kale and concentration in cottonseed oil. Particular care was observed in sampling, so that the material analyzed by the two methods was as nearly identical as possible; also the β-carotene of the vegetable was separated from the biologically inactive carotenoids by chromatography. It was found that the vitamin A value of the vegetable accounted for only 67% of the β-carotene as measured chemically. On the other hand, the carotenes extracted from the kale and fed in cottonseed oil solution gave the same vitamin A potency as did an equivalent amount of standard crystalline β-carotene fed in a like manner. This evidence suggested that "incomplete digestion of the kale, and therefore incomplete absorption of the vegetable carotene in the intestinal tract of the animal, may be an important factor causing the difference between the vitamin A values of kale obtained by chemical and biological methods." Callison and Orent-Keiles ('47) found that very large amounts of carotene in the form of cooked carrots were necessary to cure experimental human night blindness produced by a vitamin A-deficient diet. However, when the bioassay vitamin A value of the carrots was used as a basis for calculation of the human requirement, the figures conformed closely with the vitamin A requirement measured by feeding carotene in oil, frozen peas and frozen spinach (Booher, Callison and Hewston, '39; Booher and Callison, '39). These results demonstrated that neither the rat nor the human adult uses carotene from carrots as effectively as that from some other sources.

Such experiments have important implications for the development of tables of food composition to be used for the calculation of human dietary intakes and needs. Therefore, experiments have been carried out to relate quantitatively the vitamin A value and carotene content of the only two carotene-bearing root vegetables in common use, namely, carrots and sweet potatoes, and to compare them with green leafy kale.

In addition, the question has been investigated of whether or not increased fineness of subdivision of the vegetable would

facilitate absorption of the carotene and hence result in a higher bioassay value.

## EXPERIMENTAL

A single lot of California-grown carrots identified as the Imperator variety[1] bought on the Washington, D. C., market, was used in these studies. The vegetables were washed in tap $H_2O$, rinsed in distilled $H_2O$, pared thinly with a vegetable peeler and cut crosswise into ¼-inch slices. The slices were then cooked by boiling for three minutes in distilled $H_2O$, by a standardized procedure, and cooled in iced $H_2O$; small units of the cooked slices were wrapped in wax paper, packed in cartons, quick-frozen and stored at 0°C. until used. The carrots to be fed as puree were comminuted before use by passing them through a food grinder 10 times before thawing.

Similarly, one lot of market sweet potatoes of the Porto Rico variety was studied. This variety was used because of its very high carotene content (Hansen, '45; Ezell and Wilcox, '46). The potatoes were washed thoroughly in tap $H_2O$, and rinsed in distilled $H_2O$, steamed in the skins until done and transferred to the refrigerator until cool. The skins were then removed and the sweet potatoes cut into slices. Half of the slices, selected at random, were wrapped in cellophane, packaged in cartons, quick-frozen and stored at —40°C. It was necessary to store the sweet potatoes at a temperature lower than that employed for carrots in order to maintain them in the frozen state. The remaining slices were whipped to a very smooth consistency in a mechanical mixer; approximately 30-gm portions of the "mashed" sweet potato were wrapped in cellophane, packaged, quick-frozen and stored at —40°C.

The difficulties of obtaining truly representative samples for bioassay and chemical analysis from root vegetables with their uneven though symmetrical distribution of carotene were met by following the sampling procedure for carrots out-

[1] The authors are indebted to the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, for identification of the vegetable varieties.

lined by Callison and Orent-Keiles ('47). A wedge-shaped section was cut from the vegetable slice and used for bioassay; the remainder of the slice was preserved for chemical analysis by storing at 0°C.

*Biological assay*

In conducting the bioassays the criteria for depletion symptoms and length of depletion time recommended in the U. S. Pharmacopoeia (12th ed.) were employed. Weanling rats 21–28 days of age and weighing 40–49 gm were depleted of vitamin A stores on a diet consisting of hot alcohol-extracted casein 18%, cottonseed oil 10%, Osborne and Mendel salt mixture 4%, dried yeast 15%, cornstarch 53%, and viosterol to supply 3 U.S.P. units vitamin D per gram diet. Males and females were used in approximately equal numbers. When the animals declined or remained stationary in weight and displayed eye symptoms, supplementation was begun. Members of litter-mate triads received thrice weekly (a) the β-carotene standard at a level of 8.4 μg per week, (b) the vegetable at a level calculated to supply somewhat less than this amount of vitamin A value, and (c) twice this amount, respectively. Animals were weighed weekly during the 4-week assay period. To calculate the vitamin A potency of the vegetable, average growth rates of the two groups of animals that received the vegetable were related to the growth rate of the comparable group receiving the standard. When one animal of a triad failed to complete the assay period, data from the other two animals was also discarded, so that the average growth rates reported represent strict litter-mate comparisons. The sweet potatoes, both sliced and mashed, actually were fed at 4 levels because growth rates for the first week indicated that the original levels chosen were perhaps too low. However, since growth rates on the 4 levels resulted in vitamin A values which were in good agreement with one another, all the data were included in the final evaluation.

In the assays of the vegetable carotene extracts, litter-mate pairs of rats received the standard β-carotene and the amount

of extract calculated to contain the equivalent of 8.4 μg β-carotene per week, respectively. Each pair was isogenic with a triad used in the assay of either the sliced or pureed form of the vegetable.

*Chemical analysis*

At approximately 10-day intervals during the progress of the bioassays the carrots which had been reserved for chemical analysis were thawed, each slice divided in half, and a 12.5-gm aliquot of each lot of pooled halves taken for duplicate analyses. The sample was agitated for 5 minutes in the Waring Blendor with a 1:1 mixture of acetone-absolute methanol and then transferred quantitatively to a beaker and allowed to extract 24 hours longer at 0°C. It was then filtered by suction and washed with small portions of acetone-methanol, followed by three washings with petroleum ether (Skelly-solve, fraction F, purified and distilled, b.p. 40°–60°C.). The carotenols were removed by treatment with saturated methanolic KOH and sufficient distilled $H_2O$ was added to give good phasic separation. Traces of pigment which remained in the residue were extracted by repeated alternate washings with small portions of acetone-methanol and petroleum ether. The carotenoids were then completely extracted from the acetone-methanol layer with petroleum ether and the combined extracts washed free of traces of carotenols with methanolic KOH. Finally the petroleum ether extract was washed with distilled $H_2O$ until neutral, the excess water removed by "freezing out" at a low temperature, and the extract diluted to volume and stored over anhydrous $Na_2SO_4$ until used. Aliquots of this extract were taken for: (1) measurement of total carotene content; (2) chromatographing; and (3) dilution with cottonseed oil for bioassay of the carrot extract. The total carotene content of the unchromatographed extract was determined in terms of β-carotene by measuring absorption at 450 mμ with the Beckman

spectrophotometer. The chromatographing was done on ice water-cooled columns of a 1:3 MgO,[2] Hy-flo Supercel mixture. The band of α-carotene was eluted with petroleum ether and the β-carotene with 2% ethanol in petroleum ether; the band of biologically inactive carotenoids was cut from the column and eluted from the adsorbent with the ethanol-petroleum ether mixture. The several fractions were diluted to convenient volumes and read at 430, 450, and 480 mμ; calculations of carotene content were based on a standard β-carotene curve obtained with the same spectrophotometer.

Sweet potatoes were similarly sampled. The extraction procedure was carried out essentially according to the method described by O'Connor et al. ('46), except that extraction of pigments from the vegetable was accomplished by agitation in a Waring Blendor with cold 95% ethanol for 10 minutes, followed by standing in contact with 95% ethanol for 16–18 hours at 0°C. The extract was then filtered and the residue re-extracted until all color had been removed. Also petroleum ether was used in place of the iso-octane which O'Connor et al. recommend. Cooled MgO Hy-flo Supercel columns were employed in chromatographing, and the β-carotene and non-active pigments removed in separate fractions with 2% ethanol-petroleum ether. Pigments in the final solutions were measured in the same way as in the case of carrots.

## RESULTS

The total yellow pigment content of the Imperator carrot slices analyzed was equivalent to 102 μg β-carotene per gram cooked carrot, with a standard error of 9.0; the puree samples averaged 104.0 ± 9.8 μg per gram. Of these total pigments 63.2% and 62.2%, respectively, was β-carotene, 25.3% and 25.5% α-carotene, and the remainder consisted of biologically inactive carotenoids. Recoveries from the chromatographing columns averaged 90% of the total pigments. The amount of β-carotene present in this sample of Imperator carrots was somewhat higher than the 51 μg per gram re-

[2] Magnesium oxide powder, "Baker's Analyzed" reagent.

ported by Harper and Zscheile ('45) for the same variety, and the per cent of α-carotene found was considerably lower than the average of 46% for the "garden varieties" studied by the same authors.

The total yellow pigments of the sliced or mashed Porto Rico sweet potatoes averaged, in terms of β-carotene, 57.4 ± 7.4 and 54.1 ± 4.1 μg per gram vegetable, respectively. β-carotene was the only biologically active carotenoid present and constituted 81.8% of the yellow pigment in the sliced vegetable and 79.2% in the mashed vegetable. Recoveries were similar to those obtained with carrots. This ratio of β-carotene to total yellow pigment agrees with the average figure of 81.8% reported by Ezell and Wilcox ('46). However, both the total yellow pigment and the β-carotene content of the sweet potatoes analyzed in this study were in the higher range of the values reported by these authors.

The presence of the isomers of β-carotene, neo-β-carotenes B and U (called neo-β-carotene and carotenoid X by Kemmerer and Fraps) have been demonstrated in certain fresh vegetables (Beadle and Zscheile, '42; Kemmerer and Fraps, '45). These pigments are inseparable from β-carotene when adsorbed on MgO and therefore are included with the β-carotene fraction in this study.

Since neo-β-carotene B (Kemmerer and Fraps, '43) has been shown to possess about one-half and neo-β-carotene U (Kemmerer and Fraps, '45) only one-quarter to one-third the vitamin A activity of β-carotene, their presence in the β-carotene fraction in any considerable amounts would significantly alter the potential vitamin A value. Kemmerer and Fraps ('45) found no evidence of either isomer in fresh carrots and only slight traces of the B isomer in dehydrated carrots. Sweet potatoes they showed to contain carotenoid X (neo-β-carotene U) and neo-β-carotene (neo-β-carotene B) in amounts of 5.4% and 3.7%, respectively, of the total "crude" carotene. No figures for kale are available. The ratios of the absorption at 430 mμ and at 480 mμ to that at 450 mμ indicate the possible presence of isomers in the β-carotene fraction

from all three vegetables in the present study. However, since the unchromatographed extracts of carotenoid pigments from the vegetables studied gave bioassay values which agreed well with the vitamin A potency calculated from chemical analyses, it is unlikely that these two less active isomers were present in significant amounts.

Table 1 contains a summary of the growth rates of the animals receiving the several supplements, together with their standard errors. Comparison of the biological and chemical data (table 2) indicates that the proportions of biologically active carotenoids available to the rat for suboptimum growth (bioassay) are very similar for the two yellow root vegetables studied and amount to 34–41% of their potential vitamin A value, which is considerably less than the 67% availability obtained with kale (Orent-Keiles et al., '46).

There were no significant differences among the three vegetables when the carotene was extracted and fed suspended in oil; under these circumstances availability was practically complete. Fine subdivision of the carrots fed to the assay animals increased availability by only 7%; a similar procedure applied to the sweet potatoes left the availability unchanged.

Since these data were obtained, a study by Booth ('47) has been published in which the availability of carotene was investigated with 7 varieties of English carrots; chemical measurements, either with or without chromatographic separation of pigments, were compared with biological values measured either by standard rat growth technics or a liver storage procedure. Studies of fecal excretion of carotene were included. This investigator reported the vitamin A values, calculated from chemical measurements, to range from 4,900 to 93,000 international units per 100 gm wet tissue, which may be compared with a similarly derived average figure of 13,000 international units for the Imperator variety of carrots used in the present experiments. The biological values for the carrots in the English study ranged from 2,900 to 37,000 international units per 100 gm wet tissue; the percentage availability varied from 24 to 70 and tended to be less as the concentration of

TABLE 1

*Summary of bioassay data used in calculating vitamin A value of carrots, sweet potatoes and kale*

| SUPPLEMENTS | NO. OF ANIMAL TRIADS | AVERAGE WEIGHT GAINS, ± STANDARD ERROR, OF ANIMALS RECEIVING THE FOLLOWING SUPPLEMENTS: | | | | VITAMIN A VALUE [1] |
|---|---|---|---|---|---|---|
| | | Vegetable | | Carotene extract | β-carotene standard 8.4 μg/week | |
| | | "Low" level | "High" level | | | |
| | | *gm/week* | *gm/week* | *gm/week* | *gm/week* | *IU/gm* |
| Carrots, cooked: | | | | | | |
| Sliced, 0.15 and 0.30 gm/week | 33 | 9.4 ± 3.02 | 15.7 ± 3.28 | | 16.2 ± 2.70 | 45 |
| Pureed, 0.15 and 0.30 gm/week | 28 | 10.6 ± 3.37 | 18.0 ± 2.85 | | 16.6 ± 2.70 | 52 |
| Extract, ≡ 8.4 μg carotene/week | 25 [2] | | | 15.5 ± 3.67 | 16.3 ± 2.78 | .. |
| Sweet potatoes, cooked: | | | | | | |
| Sliced, 0.24 and 0.48 gm/week | 16 | 7.9 ± 3.36 | 14.8 ± 3.55 | | 14.4 ± 2.89 | .. |
| Mashed, 0.24 and 0.48 gm/week | 29 | 10.3 ± 3.14 | 14.6 ± 2.99 | | 16.1 ± 3.12 | .. |
| Sliced, 0.30 and 0.60 gm/week | 22 | 10.8 ± 3.23 | 16.6 ± 3.46 | | 15.0 ± 3.46 | 29 |
| Mashed, 0.30 and 0.60 gm/week | 24 | 10.1 ± 2.46 | 17.2 ± 3.55 | | 15.3 ± 4.01 | 26 |
| Extract, ≡ 8.4 μg carotene/week | 32 [2] | | | 15.9 ± 3.35 | 15.7 ± 3.78 | .. |
| Kale, cooked: | | | | | | |
| 0.09 and 0.18 gm/week | 19 | 7.2 ± 3.21 | 12.6 ± 3.00 | | 15.8 ± 3.51 | 60 |
| Extract, ≡ 8.4 μg carotene/week | 20 [2] | | | 12.4 ± 3.29 | 13.4 ± 2.62 | .. |

[1] Vitamin A value was calculated by plotting growth response against vegetable intake and determining the amount of vegetable equivalent to the carotene standard from this curve. In the case of the sliced carrots and kale, the growth response to the standard was somewhat greater than the response to the "high" level of vegetable; in these cases the curve was extrapolated.

[2] Litter-mate pairs instead of triads were used in the assay of the extract.

TABLE 2

*Comparison of vitamin A value of carrots, sweet potatoes and kale, as determined from carotene analysis and rat-growth bioassay* [1]

| VEGETABLE | NO. OF CHEMICAL ANALYSES | NO. TRIADS OF ANIMALS IN BIOASSAY | CAROTENE CONTENT [2] | | VITAMIN A VALUE | | VITAMIN A (BIOL. IU) / VITAMIN A (CHEM. IU) [4] × 100 |
|---|---|---|---|---|---|---|---|
| | | | α | β | Calculated from carotene [3] | Bioassay | |
| | | | *μg/gm* | | *IU/gm* | | % |
| Carrots, cooked: | | | | | | | |
| Sliced | 46 | 33 | 26 | 66 | 132 | 45 | 34 |
| Pureed | 10 | 25 | 25 | 64 | 127 | 52 | 41 |
| Extract | 10 | 25 | | | | | 96 |
| Sweet potatoes, cooked: | | | | | | | |
| Sliced | 22 | 45 | 0 | 47 | 78 | 29 | 37 |
| Mashed | 22 | 46 | 0 | 43 | 71 | 26 | 37 |
| Extract | 10 | 32 | | | | | 101 |
| Kale, cooked [5] | 64 | 19 | 0 | 54 | 90 | 60 | 67 |
| Extract | 64 | 20 | | | | | 93 |

[1] β-Carotene in cottonseed oil used as reference standard.

[2] All measurements based on wet weight of vegetable.

[3] Six-tenths μg β-carotene and 1.2 μg α-carotene equated to one international unit vitamin A.

[4] Booth ('47) has used the term "chemical IU" to distinguish between international units calculated from chemical measurements and those determined directly by bioassay. The figures in this column would therefore represent the per cent to be used in changing chemical IU to biological IU.

[5] Orent-Keiles, Callison, Schaevitz and Frenchman, '46.

carotene in the carrot increased. However, for "ordinary" carrots this percentage was 46, which is only slightly higher than the value obtained in the present study.

The results of Booth's experiments, as well as those reported here, lend support to the idea that by an accumulation of data relating chemical and biological vitamin A values obtained with well-controlled sampling and analytical procedures, it may be possible to obtain a suitable correction figure for each vegetable which can be used to reduce chemically and biologically determined vitamin A values to a common basis for use in dietary calculations. Further, the ratio of biological vitamin A values to those calculated from chemical measurements of carotene content proved to be very similar for the two yellow root vegetables studied, and only about half of that obtained from a green leafy vegetable (kale). This suggests that it may be possible to derive a single conversion factor for each general class of carotene-containing vegetables and fruits.

Additional experimentation with human subjects is necessary to determine the relative availability to the human being of carotene from various sources. The somewhat scanty information available at present indicates that whether or not the human being utilizes carotene as completely as does the rat depends upon the nature of the vitamin A active material fed (Booher, Callison and Hewston, '39; Booher and Callison, '39; Callison and Orent-Keiles, '47).

## SUMMARY

Chemical measurements of the biologically active carotenoids of the two principal yellow root vegetables, carrots and sweet potatoes, were compared with the biological vitamin A value obtained by rat growth bioassay of as nearly identical a sample of the vegetable as possible. In addition, study was made of the effect upon the availability of carotene to the animal of comminution of the vegetable and of extraction of carotene from the vegetable matrix. The carrots were of the

Imperator and the sweet potatoes of the Porto Rico variety, and both were cooked before analysis.

The carrots averaged 26 μg of α-carotene and 65 μg of β-carotene per gram of cooked vegetable; the biological vitamin A value was 34% of that calculated from the carotene content when the carrots fed were sliced, and 41% when they were pureed. The sweet potatoes averaged 44 μg of β-carotene per gram cooked vegetable and bioassay gave 37% of this value regardless of whether the sweet potato was sliced or mashed. The percentage availability of carotene from these yellow root vegetables is contrasted with 67% for cooked kale, reported earlier from this laboratory.

The feasibility is indicated of obtaining a factor for each vegetable, or possibly each class of carotene-bearing plant foods (yellow or red fruit, yellow root, green leafy, green leguminous vegetables), which could be used to convert pro-vitamin A content in terms of carotene to the equivalent international vitamin A units, derived biologically, or vice versa. A conversion of this nature is necessary in order either to combine or to compare with any degree of validity vitamin A values obtained by the two methods.

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# EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

## II. THE EFFECT OF TEMPERATURE AND HUMIDITY OF ENVIRONMENT DURING THE FIRST EIGHTEEN DAYS AFTER HATCH

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TWO FIGURES

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### INTRODUCTION

In the first paper of this series (Barott and Pringle, '47) it was shown that with chicks brooded from hatch to 9 days of age maximum growth and efficiency of feed utilization resulted when the temperature was 94°–95°F. the first day, dropping uniformly to 88°F. on the 9th day. This research has been extended in the present experiment to cover the first 18 days after hatch and to include the effect of humidity.

### PROCEDURE[2]

The work was continued in the small houses with environmental control described in the earlier paper. The same

[1] Acknowledgment is made of the assistance of James Jackson, who aided in the general routine of conducting these experiments.

[2] One important detail should have been mentioned in the original paper. As stated, all chick cages were equipped with wire mesh floors. As made by the manufacturers these floors are from 1½" to 2" below the top of the feed trough. This is not conducive to prompt feeding of day old chicks. Therefore, in all experiments currently reported this floor was raised to the level of the feed troughs for the first few days and thereafter gradually lowered, reaching the original position at about the 10th to 12th day after hatch.

equipment, feed formulas and controls were used and the routine followed was identical. The chicks, Rhode Island Reds, were obtained from the same flock and 30 chicks were used in each experiment as before. Mortality was practically nil.

As was explained in the first paper, all chicken weights were corrected for the amount of extraneous matter in the system. To determine this amount, 30 chickens were dissected daily from the 9th to 18th day of age. The contents of the gizzard and intestinal tract were removed and weighed separately and an average weight for the 30 chickens obtained. (All weights of chickens used in these experiments were obtained 4 hours after the last feeding period; consequently there was no feed in the crops, and normally all yolk had been utilized by the 7th day.)

Values obtained for undigested food material in the system increased lineally from 7.20 gm on the 9th day to 11.30 gm on the 18th day.

TABLE 1

*Temperatures on the 18th day after hatch for the 53 experiments*

| Number of experiments | 3 | 3 | 10 | 11 | 10 | 3 | 5 | 4 | 4 |
|---|---|---|---|---|---|---|---|---|---|
| Temperature on 18th day (F°) | 86 | 83 | 82 | 81 | 80 | 79 | 78 | 74 | 70 |

## RESULTS AND DISCUSSION

### *Effect of temperature*

The temperature for the first 9 days after hatch was that reported as the optimum in the first paper: i.e., 94°–95°F. at the start, dropping uniformly to 87°–88°F. on the 9th day.

To determine the optimum temperature from the 9th to the 18th days, 53 experiments were conducted with a total of 1590 chickens. The temperatures on the 18th day were as shown in table 1.

On the basis of comparative analyses of the results obtained for all experiments conducted within a given temperature range, the gain in weight of the chickens between the 9th and 18th days in that range of temperature was computed as a

per cent of the weight on the 9th day. The gain was also computed for each lot of chickens for 18 days (from the start to the 18th day) as a per cent of the initial weight. The results so obtained for each temperature range were plotted, as in figure 1, and curves drawn through the plotted points.

The numeral at each point designates the number of experiments made to obtain that point: e.g., a point with the figure 10 indicates that 10 experiments of 30 chicks each, or a total of 300 chickens, were used to obtain a mean value for that point.

The point is plotted at the mean temperature prevailing during the period investigated for the particular group.

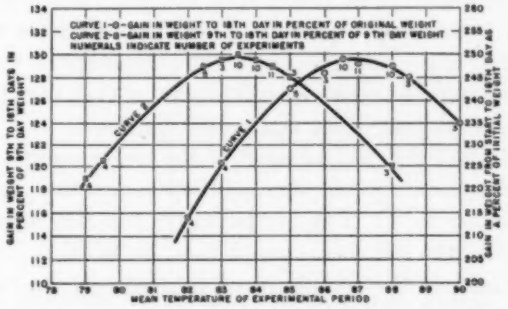
CURVE 1—GAIN IN WEIGHT TO 18TH DAY IN PERCENT OF ORIGINAL WEIGHT
CURVE 2—GAIN IN WEIGHT 9TH TO 18TH DAY IN PERCENT OF 9TH DAY WEIGHT
NUMERALS INDICATE NUMBER OF EXPERIMENTS
CURVE 2
CURVE 1
MEAN TEMPERATURE OF EXPERIMENTAL PERIOD

Fig. 1 Effect of temperature of environment on growth.

Curve 2, which represents growth between the 9th and the 18th days, shows a definite variation due to temperature. The maximum growth occurred when the mean temperature was 83.5°F. (87°F. on the 9th day and 80°F. on the 18th day) and resulted in a 130% increase in the last 9 days over the weight on the 9th day. The gain in weight was less at other temperatures and became less and less the further the temperature deviated above or below that found to be associated with maximum growth.

Curve 1, which gives the growth in 18 days as a per cent of the initial weight, shows that the maximum growth occurred at a mean temperature of 87°F. (94°F. at the start and 80°F.

on the 18th day) and equaled a 249% increase in weight. In other words, the chickens were 3½ times as heavy on the 18th day as they were when the experiment started.

For the 18 days as a whole, the maximum growth occurred at a mean temperature of 87°F.; this would then be the temperature on the 9th day, as this is the mean time for the 18 days.

The efficiency of the chicken (grams gain in weight divided by grams of feed eaten) follows the same trend as the curves in figure 1, the greatest efficiency occurring at the temperature where the growth was greatest and becoming less and less as the deviation from this temperature increased.

The data reported in this paper are the results of experiments not previously reported. These experiments were continued for 18 days and therefore the temperature for the 9th day was established more accurately than that for the 9 days previously reported. For optimum conditions the temperature should be 94°F. at the start of the experiment. It should be reduced uniformly each day, so the temperature on the 18th day should be 80°F. The temperature for the mean of the 18 days (i.e., the 9th day) would then be 87°F.

Table 2 shows the temperature that should prevail at the start of each day.

In the previous paper already cited reference was made to the work of Kleiber and Dougherty ('33). They found that for chickens kept at a constant temperature from the 5th to the 15th days, the best growth occurred at the lowest temperature they studied, i.e., 21°C. (70°F.) and that it became less and less as the temperature was increased to 40°C. (104°F.). Table 2 shows the temperature required each day for optimum growth. As determined in the present investigation, this temperature was 90.7°F. on the 5th day and 82.5°F. on the 15th day, which is considerably higher than that reported by Kleiber.

After the range of temperature for maximum growth had been determined (fig. 1), the 23 experiments conducted within

this range were analyzed to determine the values from which the curves in figure 2 were constructed.

Curve 4, which shows the increase in weight as a per cent of initial weight, is the typical growth curve for chickens. Growth starts slowly for the first 4 or 5 days after hatch and then accelerates rapidly until about the 10th or 12th day, after which it seems to be practically linear until the 18th day. Between the 12th and the 18th days the chicken is increasing in weight about $\frac{1}{3}$ of its original weight in each two days.

TABLE 2

*Temperature at the start of each day*

| AT START OF DAY | TEMPERATURE | AT START OF DAY | TEMPERATURE |
|---|---|---|---|
| | *F°* | | *F°* |
| 1 | 94.0 | 10 | 86.6 |
| 2 | 93.2 | 11 | 85.8 |
| 3 | 92.4 | 12 | 84.9 |
| 4 | 91.5 | 13 | 84.1 |
| 5 | 90.7 | 14 | 83.3 |
| 6 | 89.9 | 15 | 82.5 |
| 7 | 89.1 | 16 | 81.6 |
| 8 | 88.2 | 17 | 80.8 |
| 9 | 87.4 | 18 | 80.0 |

Curve 1, showing the increase in weight in grams, seems to be practically linear between the 9th and the 18th days, the increase in weight each day over that of the previous day being approximately 0.7 gm.

Curves 2 and 3 show, respectively, the amounts of feed and water consumed per chicken per day. These curves are nearly linear although there seems to be a small increase from day to day. From all observed data in these investigations it appears that the ratio of water drunk to feed eaten is practically constant. This is indicated in curve 6, which shows the ratio of feed eaten to water drunk. This curve starts at 0.61, rises rapidly to 0.63 on the 6th day, and then rises more slowly to slightly over 0.64 on the 18th day. The chickens drank approximately 1.55 gm of water for each gram of feed eaten.

Curve 5 shows the efficiency of feed utilization: i.e., gain in weight per gram of feed eaten. The values from which the curve was plotted were obtained, as in the previous paper, by dividing values taken from curve 1 (daily gain in weight) by values taken from curve 2 (daily feed eaten, in grams) at the corresponding time.

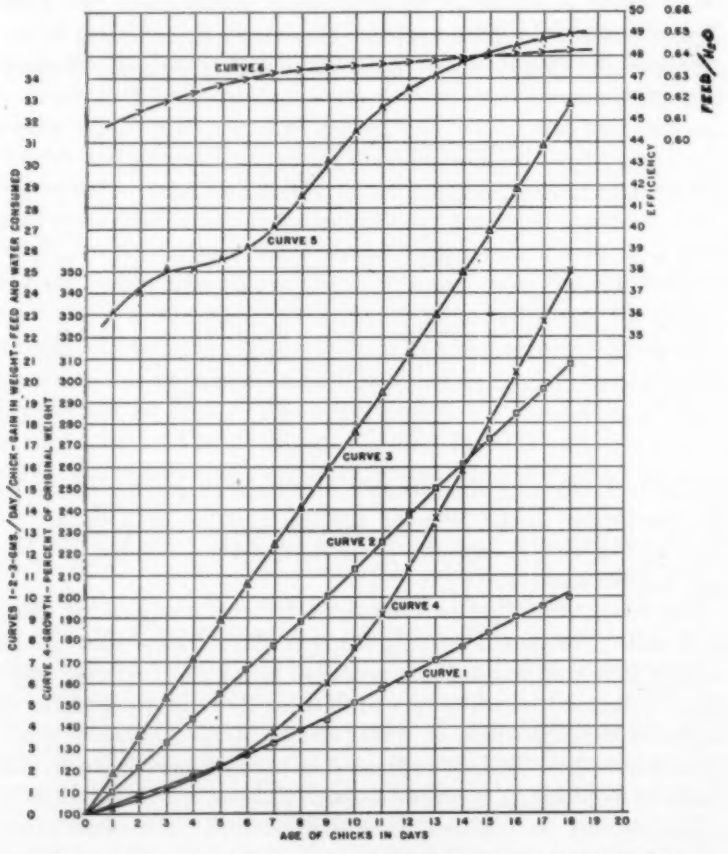


Fig. 2 Growth and feed and water consumption of chickens from hatch to 18 days of age.

The efficiency rose from a value of 0.43 on the 9th day to 0.49 on the 18th day. The increase in efficiency is most rapid between the 6th and the 10th days, and gets less and less each day, from the 10th to the 18th. Between the 7th and 9th days it rose from 0.40 to 0.43, while from the 17th to the 18th it rose from 0.485 to 0.490.

The figure of 0.49 for efficiency of feed utilization on the 18th day (curve 5) is higher than that found in the results published by other investigators. We feel that this higher value is more correct because of the unusual care taken in the present work to measure accurately the amount of feed eaten.

The change in weight of the troughs from day to day does not indicate the amount of feed eaten as accurately as might be supposed. There is a change in weight due to moisture gained or lost from the feed, depending upon whether the hover is at a higher or lower humidity than the place of feed storage. But the greatest correction to the loss in weight of the feed trough from day to day is due to the feed which is thrown from the trough by the chickens. If the conditions of the experiment are such that this waste feed cannot be accurately determined, then all values for feed eaten are too great and all values for efficiency too low.

The correction for change in weight of the feed troughs due to moisture gained or lost to the atmosphere was determined by exposing a trough, identical with the feed troughs and containing the same amount of feed, to the atmosphere of the brooder room but out of the reach of the chickens. The change in weight of this trough was obtained each day and applied as a correction factor to the food eaten.

The correction so obtained may be too small because the surface of the feed in the check trough was undisturbed, while that in the feed troughs was continuously changing due to the chickens feeding. However, this correction is not large, amounting to from one to 4 gm per day. During the first few days, when the chickens are small and not eating so much as later, this amount is important, as a three-gram

change in weight amounts to about 3% or more of the feed eaten. This correction, as a per cent of total feed eaten, becomes less and less important as the chickens grow older and the consumption of feed increases.

The loss due to feed that was thrown from the trough by the chickens was determined by carefully collecting the waste feed and weighing it. It is important that this correction be determined accurately, because the amount of feed thrown from the trough may in extreme cases equal as much as 50% of the feed eaten.

TABLE 3

*The effect of humidity on growth*

| RELATIVE HUMIDITY | NUMBER OF EXPERIMENTS | INCREASE IN WEIGHT | | |
|---|---|---|---|---|
| | | 0 to 9th day as a per cent of initial weight | 0 to 18th day as a per cent of initial weight | 9th to 18th day as a per cent of weight on 9th day |
| % | | % | % | % |
| 35 | 3 | 48 | 134 | 245 |
| 50 | 3 | 52 | 130 | 244 |
| 55 | 8 | 50 | 133 | 240 |
| 60 | 9 | 52 | 126 | 240 |
| 65 | 10 | 52 | 128 | 245 |
| 70 | 1 | 50 | 126 | 236 |
| 75 | 3 | 51 | 127 | 242 |

## *Effect of humidity*

A total of 37 experiments (1110 chicks) were conducted with relative humidities ranging from 35% to 75%. Little, if any, difference in growth was noted, as can be seen by the results shown in table 3. The temperature in these experiments ranged from 93°–95°F. at the start to 78°–83°F. on the 18th day.

## SUMMARY

Chickens were housed in a controlled environment without a hover for the first 18 days after hatch, to determine the optimum temperature for growth and efficiency of feed utilization. Thirty chickens were used for each experiment and a total of 53 experiments were performed. Equipment, feed formulas and routine of procedure were the same as for

work reported in a pevious paper by the present authors ('47).

The temperature during the first 9 days was kept at the optimum as reported in the first paper: i.e., 94°–95°F., at the start, and then dropped uniformly to 87°–88°F. on the 9th day. For the period from the 9th to the 18th days the temperature was varied with different lots of chickens from a temperature of 87°–88°F. on the 9th day to temperatures ranging from 86°–70°F. on the 18th day.

Maximum growth over the period from the 9th to the 18th day was found when the temperature dropped uniformly from 87°F. on the 9th day to 80°F. on the 18th day. The growth under these conditions equaled a 130% increase in weight over the weight on the 9th day.

The efficiency of feed utilization rose from a value of 0.43 on the 9th day to 0.49 on the 18th day. Both growth and efficiency became less as the temperature was varied either way from the range noted for maximum growth, and the greater the variation the greater the difference.

The amount of feed consumed increased approximately 1 gm per day per chick. On the 18th day after hatch the chicks consumed approximately 2.02 gm of feed for each gram gain in weight and drank approximately 1.55 gm of water for each gram of feed eaten.

Humidity seems to make little if any difference in growth within the range here reported.

## LITERATURE CITED

BAROTT, H. G., AND EMMA M. PRINGLE 1947 The effect of environment on growth and feed and water composition of chickens. J. Nutrition, *34:* 53–58.

KLEIBER, M., AND J. E. DOUGHERTY 1933 The influence of environmental temperature on the utilization of food energy in baby chicks. J. Gen. Physiol., *17:* 701.

# ERRATA

The Journal of Nutrition, vol. 36, no. 1, July 1948.

N. Glickman, H. H. Mitchell, E. H. Lambert and R. W. Keeton

Pages 49 and 51, equations (1), (2), (3), and (4) should read:

$$S_p = 173 - 217.3e^{-0.230161t} \quad (1) \qquad \frac{dS_p}{dt} = 54.35e^{-0.230161t} \quad (3)$$

$$S_c = 108 - 118.5e^{-0.260781t} \quad (2) \qquad \frac{dS_c}{dt} = 31.25e^{-0.260781t} \quad (4)$$

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The Journal of Nutrition, vol. 36, no. 5, November 1948.

Barnett Sure and Frances House

Page 599, line 9 should read:

"and rice in the non-milled state showed essentially the same values"